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Use of Eubacterium coprostanoligenes to decrease plasma cholesterol concentration in hypercholesterolemic rabbits and the cholesterol content of fermented meats

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Use of *Eubacterium coprostanoligenes* to decrease plasma cholesterol concentration in hypercholesterolemic rabbits and the cholesterol content of fermented meats

by

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DEDICATION

To the people who contributed their love, sacrifices, discipline, support and humble prayers; my father and mother Mr. and Mrs. Obediah Augustus Madden and family in Jamaica, West Indies, and my Poisonous Plants professor and his dear wife, Dr. and Mrs. Robert L. Judkins in Tuskegee Alabama

....and to the memory of my landlord and his wife, Mr. Wilson and Mrs. Madge Richburg (Tuskegee, Alabama).

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GENERAL INTRODUCTION

Epidemiological studies have shown strong correlation of plasma cholesterol concentration and risk of coronary heart disease (Noruma et al., 1992). The incidence of coronary heart disease is rare in populations with plasma cholesterol lower than 5.2 mmol/l and risks of coronary heart disease increase when plasma cholesterol is higher (Isles and Hole, 1992). Informed consumers worldwide prefer low-fat food, which should result in lower plasma cholesterol concentration and thus lower risk of coronary heart disease (Schroder and Baer, 1990). Cholesterol content of animal-derived foods is a primary public concern as there is considerable evidence that suggests that plasma cholesterol plays an important role in the development of atherosclerosis and coronary heart disease (Dehal et al., 1991). The fatty acid composition of adipose tissue reflects that of the diet and is an objective biochemical indicator of the kind of fats consumed for several years (Seidelin et al., 1992). The amount and type of fat in the diet affect blood lipid concentrations (Norum, 1992).

Treatment of hypercholesterolemia by diet or by drugs decreased the incidence of coronary heart disease (Katan, 1992). Lipid-lowering drugs may pose adverse side effects to patients with hypercholesterolemia and there is need for development of technology for safer applicable methods of

treatment of hypercholesterolemic patients and to decrease the cholesterol content of foods.

The hypothesis for these studies is that consumption of foods containing *E. coprostanoligenes* or ingestion of an effective dose of bacteria and colonization of the intestinal tract of by this cholesterol-reducing microbe will result in conversion of dietary and endogenous cholesterol to coprostanol, which is poorly absorbed from the intestinal tract of humans and animals. These investigations were conducted to determine the potential for the use of *Eubacterium coprostanoligenes* to decrease plasma cholesterol concentration in hypercholesterolemic rabbits as a model for human hypercholesterolemia and to decrease the cholesterol content of fermented meats as a model for decreasing the cholesterol content of foods.

Dissertation Organization

This dissertation consists of acknowledgements, a general introduction containing a review of literature, two separate papers, and general conclusions. The references cited in each paper are listed as REFERENCES at the end of each paper.

Literature Review

It is hypothesized that there is a causal association between serum cholesterol concentration, dietary fat, and coronary heart disease in humans, and it is believed that the risk of coronary heart disease should be decreased substantially when serum cholesterol concentration is lowered (Ravnskov, 1992). Incidence of coronary heart disease is rare in populations with plasma cholesterol lower than 5.2 mmol/1, and risks of coronary heart disease increase when plasma cholesterol is higher (Isles and Hole, 1992). Epidemiological studies have indicated that there is a high correlation of plasma lipid concentrations, especially lipoprotein (LDL)-cholesterol, with risk of coronary heart disease (Noruma et al, 1989; Noruma et al., 1992).

Cholesterol content of animal-derived foods is a primary public concern as there is considerable evidence that suggests that plasma cholesterol plays an important role in the development of atherosclerosis and coronary heart disease. Cholesterol is required for several important biological functions in animals: precursor for biosynthesis of bile acids, corticosteroids and sex hormones; regulation of membrane activity and transport of lipid from the intestines to tissues; and proliferation of a variety of cells (Dehal et al., 1991).

In a pig model on restricted intake, it was observed that

cholesterol-deprived pigs with similar caloric intake to cholesterol-fed pigs had a net weight loss, whereas cholesterol-fed animals had a net weight gain (Neu et al., 1987). Studies conducted on the effect of soy versus beef diets on blood and tissue cholesterol and body composition of growing swine showed that no significant differences were observed in total plasma cholesterol or liver cholesterol concentrations. In soy-fed pigs, significantly greater cholesterol concentrations were found in the viscera excluding liver ($p<.001$), aorta ($p<.04$), carcass ($p<.001$) and whole body (liver + other viscera + carcass) ($p<.001$) (Walsh et al., 1983). In hypercholesterolemic pigs fed a western type diet composed of baked beans (*Phaseolus vulgaris*) at 100, 200, and 300 g/kg reduced plasma total cholesterol by 5.3, 20.2 and 35.6%, respectively (Costa et al., 1993). In miniature pigs fed fish oil and corn oil, triacylglycerol and cholesterol in plasma and VLDL were significantly reduced (Huff et al., 1993).

In studies conducted on the effect of a bean diet on biliary lipid secretion, serum cholesterol concentration and hepatic cholesterol metabolism in rats, the results showed increased output of biliary cholesterol and biliary phospholipid, whereas biliary flow and biliary salts output remained in the normal range. Total serum and very-low-density lipoprotein (VLDL) cholesterol concentrations decreased

significantly (27% and 50%), whereas hepatic cholesterogenesis increased 170% in the bean-fed animals. Newly synthesized cholesterol contribution to total biliary cholesterol increased by 200% and was preferentially channelled to the biliary cholesterol secreted in these rats (Rigotti et al., 1989). Elucidation of intrahepatic determinants of biliary cholesterol output was done in rats by feeding 1% diosgenin for one week. The results showed that biliary cholesterol output and saturation increased by 400%, whereas bile flow, biliary bile salts, phospholipids and protein outputs remained in the normal range (Nervi et al., 1984). In a cholesterol model system study of cholesterol movements between plasma and organs in rats following a single injection of red cells containing free (unesterified) [^{3}H] cholesterol, results showed that cholesterol movements between plasma and different organs occurred mainly through intense exchange of free cholesterol resulting in a low net flux (Magot et al., 1987).

Determination of the amount of LDL-cholesteryl ester converted to biliary steroids in rats after a single intravenous or pulse injection of LDL-[^{3}H]cholesterol linoleyl ester, followed by constant infusion to maintain plasma radioactivity showed that although a substantial (53-61 ug/h) amount of cholesteryl esters were released into the liver during catabolism, only a small fraction (0.8-1.90 ug/h) was

found in biliary steroids (Bhattacharyya et al., 1986). Absorption studies of saturated analogues of cholesterol by rat small intestine showed that cholestanol was absorbable whereas coprostanol was unabsorbable because of differences in uptake and esterification (Bhattacharyya, 1986).

In a study of the relationship between rates of hepatic sterol synthesis and rates of hepatic LDL uptake (clearance) in animals with high (rats), low (female hamsters) and very low (male hamsters) basal rates of hepatic sterol synthesis, it was found that in rats and female hamsters fed cholesterol or cholestyramine, rates of hepatic sterol synthesis varied over 110-fold range, whereas rates of hepatic LDL clearance and plasma LDL-cholesterol concentrations remained unchanged. In male hamsters, cholestyramine feeding increased rates of hepatic LDL uptake by 2.5-fold and was associated with a 50% reduction in plasma LDL-cholesterol concentrations (Spady et al., 1985).

The classification of lipoproteins by lipidologists has been done by methods of separation with the assumption that the separation techniques were based on physicochemical properties and had physiological significance. The two methods were based on flotation in a prescribed density solution, very-low-density lipoproteins (VLDL), LDL and HDL, and in a charged prescribed buffer solution (pre-beta migrating lipoproteins, beta-migrating lipoproteins and alpha migrating

lipoproteins). The density of lipoproteins is dependent on the makeup of the lipoprotein particle and on the types of molecular components and it is sensitive to changes in triglyceride content when indexed by the remaining lipids. Lipids are transported in plasma by forming lipoprotein particles (Zech et al., 1986).

In sheep, rabbit, guinea pig and wild boar, most of the cholesterol (70-76%) was in the beta-lipoprotein fraction, whereas in horse (60%), mink (60%), fox (65%), goat (70%) and cattle (73%) the HDL fraction was the main carrier of cholesterol (Vitic and Steveanovic, 1993).

The three long-chain fatty acids most commonly found in neutral fats in the human body are similar to the major constituents of fats in foods: (a) stearic acid, which has 18-carbon atoms and is fully saturated with hydrogen atoms, (b) oleic acid, which has 18-carbon atoms and one double bond in the middle of the chain, and (c) palmitic acid, which has 16-carbon atoms and is fully saturated (Guyton, 1966). The fatty acid composition of adipose tissue reflects that of the diet and is an objective biochemical indicator of the kind of fats consumed for several years (Seidelin et al., 1992).

The amount and type of fat in the diet can affect blood lipid concentrations. A recent study from Taiwan showed that large amounts of monounsaturated fat in the diet raised plasma lipids (Norum, 1992). The effects of saturated fats on plasma

cholesterol are seen only with saturated fats having chain lengths of 12, 14 and 16 carbons atoms, which show increase in total cholesterol mainly in the concentrations of LDL. Shorter chain fatty acids have little effect on plasma LDL (Norum, 1992). Recent investigations have shown that stearic acid failed to increase LDL-cholesterol concentration in plasma, whereas oleic acid was as effective as linoleic acid in lowering plasma LDL-cholesterol when substituted for palmitic acid (Mattison and Grundy, 1985).

The hypocholesterolemic effect of dietary polyunsaturated fat in guinea pigs may have resulted from redistribution of plasma cholesterol to body tissues by increased tissue LDL receptors (Minocha et al., 1988). In a study conducted in rats on the output of bile and biliary components (biliary salts, phospholipids and cholesterol), during the first hour after bile duct catheterization it was found that feeding a commercial diet or semi-purified diets containing 7% or 20% corn oil or 20% lard or mutton tallow, the output of cholesterol was augmented when the percentage of corn oil was increased from 7% to 20% (Bouquillon and Clement, 1979).

In studies relating to the effects of fat unsaturation and fatty acid composition on the development of experimental atherosclerosis in rabbits after feeding cholesterol or an atherogenic or cholesterol-free semipurified diet, it was found that the severity of atherosclerosis was inversely

related to the level of fat saturation. Cocoa butter was found to be less atherogenic than expected, whereas peanut oil, which is relatively unsaturated, was atherogenic for rats, rabbits and monkeys (Kritchevsky, 1991).

Structures of Cholesterol and Related Sterols

The structures of cholesterol and related sterols consist of a cyclopentanophenanthrene ring, which can be synthesized from a simple precursor such as acetate by most mammalian cells. Liver and small intestines are the most dominant in rate of cholesterol synthesis (Boyd, 1975). Structures of sterols related to cholesterol are shown Figure 1 and 2 (Boyd, 1975). A proposed model for metabolism of cholesterol by microorganisms and enzymes is shown in Figure 3 (Macdonald et al., 1985). Figure 4 shows proposed mechanisms for the formation of coprostanol from cholesterol (Bjorkheim and Gustafsson, 1971).

Metabolism and Distribution of Cholesterol

Multicompartmental analysis of cholesterol metabolism in humans was studied in patients with total bile fistula; radiolabelled mevalonic acid and [^{14}C]cholesterol were administered simultaneously. The results indicated that newly synthesized hepatic cholesterol contributed 31% and 20% to bile acid and biliary cholesterol, whereas plasma free

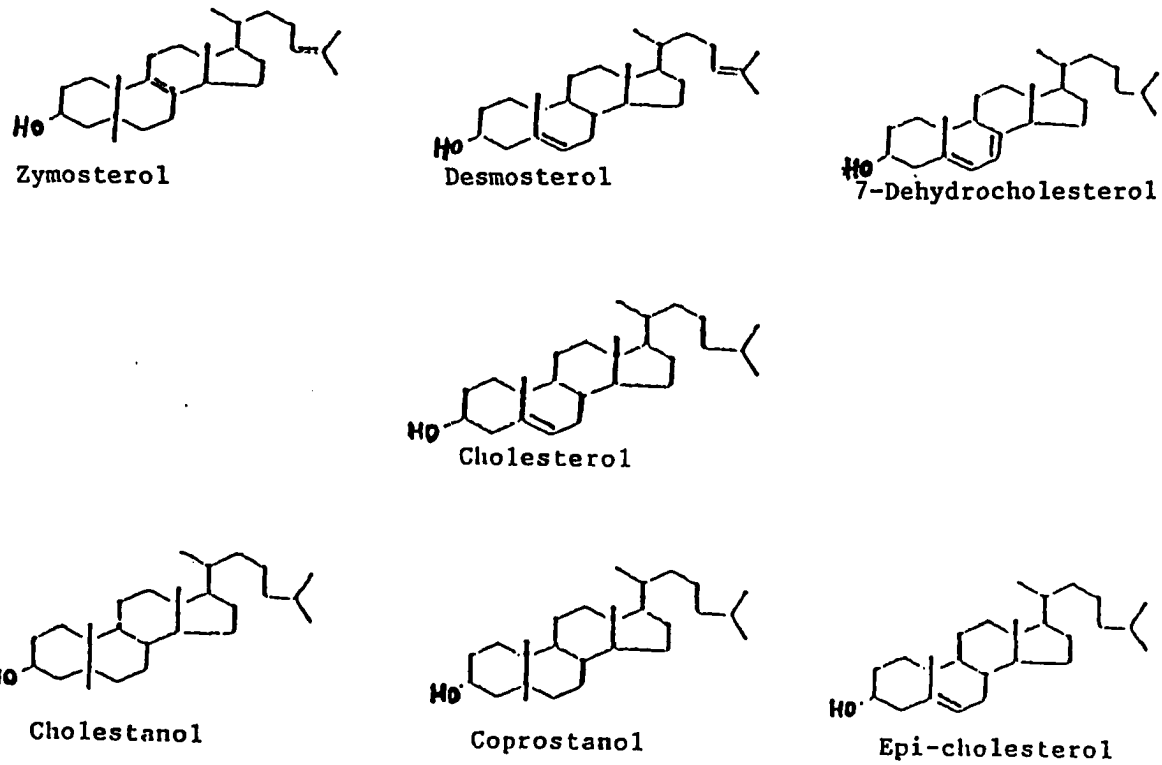

Figure 1. Structures of cholesterol and related sterols which may be present in tissues (Boyd, 1975)

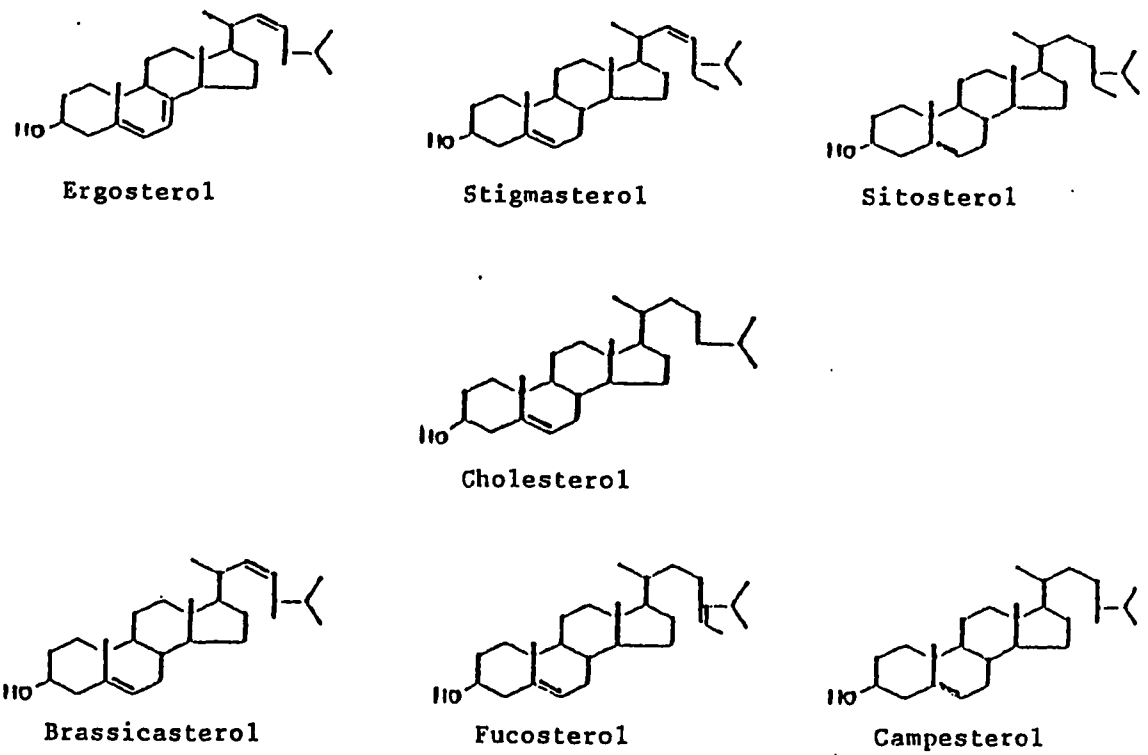

Figure 2. Structures of cholesterol and related sterols which may be present in various plants and unicellular organisms (Boyd, 1975)

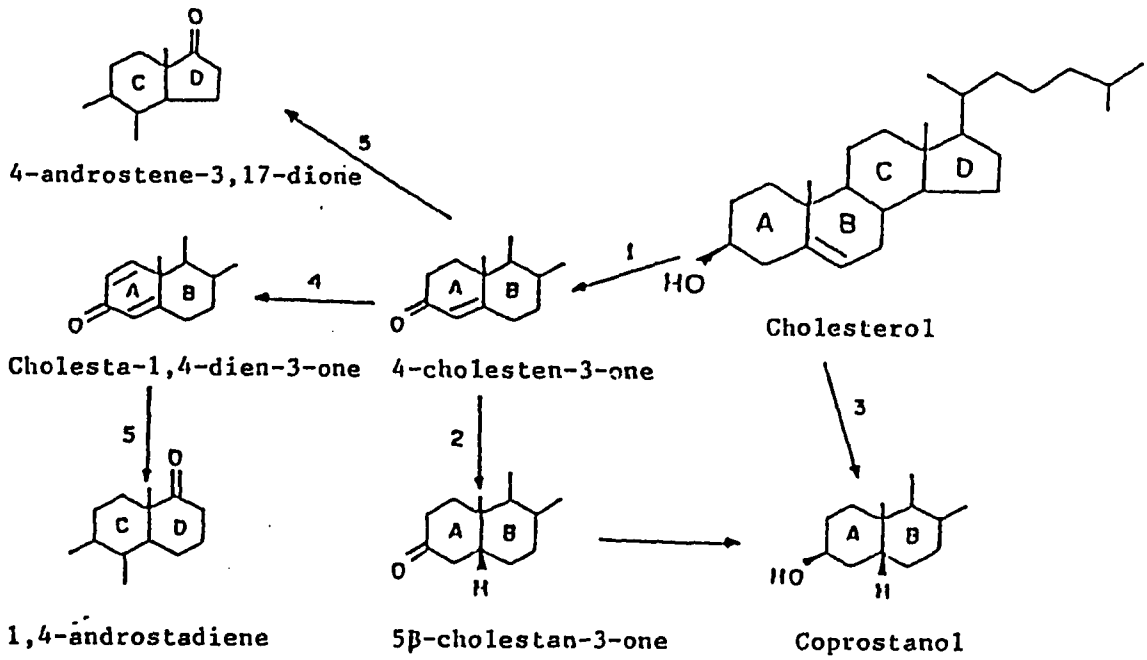

Figure 3. Model for metabolism of cholesterol by microorganisms and enzymes: 1) *E. coli*, *Eubacterium 21408*, cholesterol dehydrogenase; 2) *Eubacterium*, *Bacteroides*, *Clostridium*, *Bifidobacterium*, Δ^4-NDH; 3) *Eubacterium*, Δ^5-NDH; *E. coli*, 4) Δ^1-Dehydrogenase; 5) human fecal flora *E. coli*, desmolase (Macdonald et al., 1983)

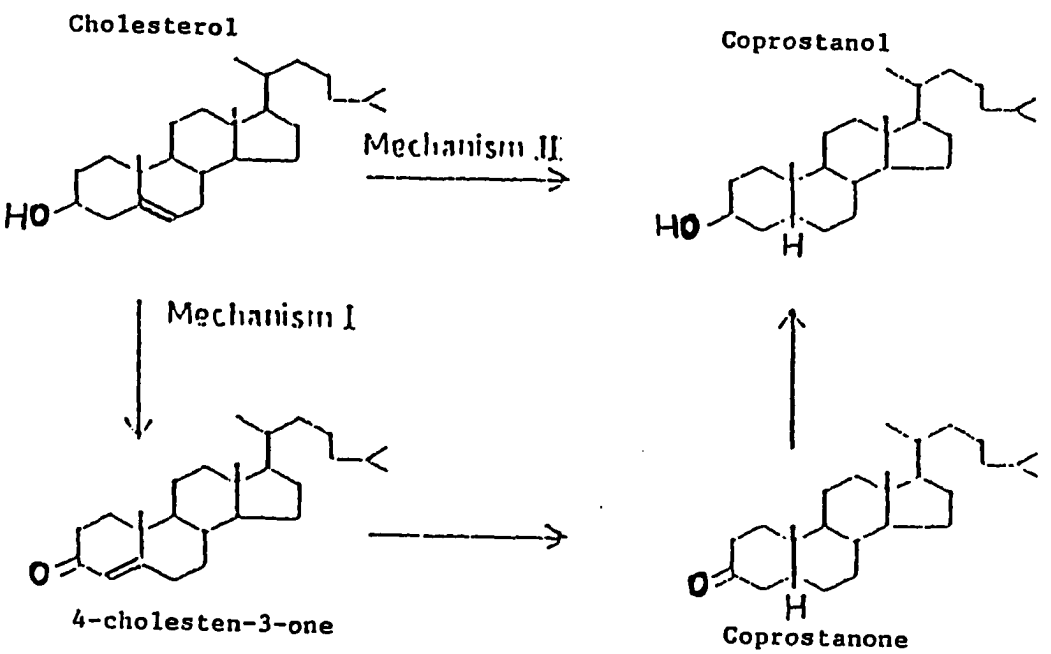

Figure 4. Proposed mechanisms for the formation of coprostanol from cholesterol (Bjorkheim and Gustafsson, 1971)

cholesterol made a small contribution (11%) to bile acid (Schwartz et al., 1978). Elucidation of cholesterol dynamics in membranes is essential in the understanding of cellular cholesterol accumulation and loss. Cholesterol is not randomly distributed in model or biologic membranes, but appears to be organized in structural and kinetic cholesterol-rich and poor domains, which can be observed histochemically and can be physically isolated from epithelial cell surface membranes (Table 1) (Schroeder et al., 1991).

Lipids make up a major fraction of each class of lipoproteins, which are transported in plasma. These macromolecular complexes are constructed so that the more polar constituents are on the lipoprotein surface (S) forming a boundary layer which interacts directly with the aqueous environment. The less polar moieties make up the core (C) which is relatively isolated from the bulk plasma. Human plasma lipoproteins are a polydisperse collection of macromolecular complexes ranging from 7 to 160 nm and contain the moieties shown in Table 2 (Zech et al., 1986).

Results from an investigation on the effect of dietary fats on the fluidity of human HDL suggest that the percentages of triacylglycerol and oleic acid in phospholipids of HDL have a fluidifying effect on these lipoproteins (Sola et al., 1990).

The investigation of the action of lecithin-cholesterol acyltransferase (LCAT, EC 2.3.1.43) on different pig

Table 1. Asymmetric distribution of cholesterol in cells

Type
Intracellular
Plasma membrane>microsome>mitochondria
Intramembrane-transbilayer
Intramembrane-lateral
Macroscopic domains
Microscopic domains

Schroeder et al., 1991.

Table 2. Components of human plasma lipoproteins

Moiety	Location	Polar
Triglyceride	C	-
Cholesterol	S	+
Cholesterol esters	C	-
Phospholipid	S	+
Apolipoprotein[a]	S	+
Sphingomyelin	S	+

C = core
S = surface
- = less polar
+ = more polar
[a](10 or more)
Zech et al., 1986.

lipoprotein classes showed that in the absence of active LCAT there was a substantial transfer of free cholesterol from LDL to HDL and a small transfer of cholesteryl esters in the opposite direction. The loss of free cholesterol was immediate in all three lipoproteins and the presence of LCAT was more prominent in LDL and was proportional to the newly synthesized cholesteryl esters incorporated in each fraction (Knipping et al., 1987). It is hypothesized that cholesteryl esters formed

in HDL by LCAT mediate the return of cholesterol from extrahepatic tissues to the liver for excretion and reutilization (Goldberg et al., 1991).

The circulating concentrations of plasma HDL may be an important determinant of plasma cholesteryl ester transfer activity, through the function of HDL-deprived lipids transfer inhibitor protein (Tollefson et al., 1988). LCAT is the enzyme that catalyzes the esterification of free cholesterol in plasma lipoproteins (Bujo and Saito, 1993). Investigation of the mechanism for the regulation of cholesterol esterification by LCAT in pig plasma showed that reaction with phosphatidylcholine small unilamellar vesicles, cholesterol, water, diacylglycerol, and lysophosphatidylcholine were all potent acceptors of acyl group cleaved from the sn-2 position of egg phosphatidylcholine, generating cholesteryl ester, free fatty acid, triglyceride, and phosphatidylcholine, respectively (Czarneck and Yokoyama, 1993). LCAT activity was positively correlated with plasma total cholesterol in the normocholesterolemic pigs, whereas it was negatively correlated in the hypercholesterolemic group (Lacko et al., 1992).

In a rabbit model, dietary cholesterol downregulates hepatic LDL receptor and treatment with 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA, 1.1.1.34) reductase inhibitors partly restored the expression (Roach et al.,

1993). The HMG CoA reductase reaction is a rate-limiting step in cholesterol biosynthesis (Black et al., 1988). Three components involved in cholesterol homeostasis, the B/E (LDL) receptor, HMG CoA reductase and acyl-coenzyme A: cholesterol acyltransferase activity (ACAT, EC 2.3.1.26) were characterized in the livers of hypercholesterolemia-resistant rabbits. Resistant rabbits manifested several quantitative differences in cholesterol metabolism and regulation (Loose-Mitchell et al., 1991).

In piglets fed sow milk or infant formula with or without addition of cholesterol, liver total cholesterol, cholesteryl esters, biliary bile acids, and phospholipid concentrations were higher and liver HMG CoA reductase activity and plasma lithosterol:cholesterol ratio were significantly lower in piglets fed the infant formula with cholesterol than in piglets fed the infant formula without cholesterol (Rioux and Innis, 1993).

In cholesterolemia-resistant rabbits, less dietary cholesterol was absorbed and it appeared that a major mechanism controlling plasma cholesterol concentrations involved the rate of cholesterol conversion to bile acids (Overturf et al., 1990). New Zealand White rabbits resistant to hypercholesterolemia excreted more lithocholic and deoxycholic acid than cholesterol-fed normal rabbits. A higher than normal 7 alpha-hydroxylase activity and cholesterol 7

alpha-hydroxylase mRNA was observed in livers from the resistant rabbits versus normal rabbits (Poorman et al., 1993). A decrease in the activity of 7 alpha-hydroxylase, the rate limiting enzyme in the catabolism of cholesterol to bile acids, resulted in increased biliary cholesterol concentration and supersaturation of bile. Ascorbic acid affects the rate-limiting step in the catabolism of cholesterol in guinea pigs and many human groups have the risk for cholesterol gallstones that are associated with decreased ascorbic levels (Simon, 1993).

The description of a typical three pool system of cholesterol dynamics in mammals is shown in Figure 5. The system contains plasma, liver, small intestines and erythrocytes in pool 1 where rapid equilibration of cholesterol occurred between tissues after administration of isotopically labelled cholesterol (Boyd, 1975). In pool 2, slower equilibration of cholesterol than in pool 1 occurred between plasma and tissues such as cardiac and skeletal muscles. The slowest equilibration of cholesterol occurred in pool 3 which contains tissues such as brain and nerve and others (Boyd, 1975).

The interrelationship between liver, small intestines, plasma and extrahepatic tissues regarding cholesterol exchanges is shown in Figure 6. Liver and small intestines have the ability to synthesize cholesterol from precursors

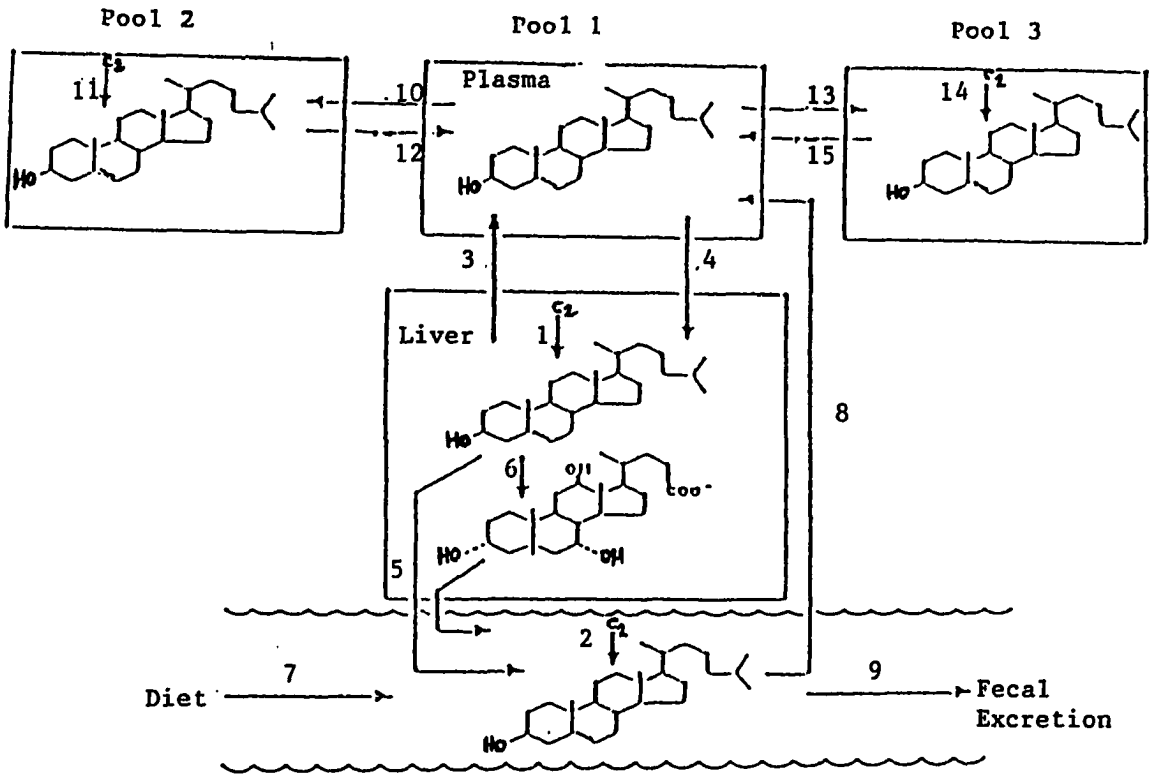

Figure 5. Three pool system of cholesterol dynamics in mammals: pool 1, plasma, liver, small intestine, erythrocytes; pool 2, cardiac and skeletal muscles; pool 3, brain and nerves; 1 = rate of synthesis in liver; 2 = rate of synthesis in small intestine; 3 = rate of transference from liver to plasma; 4 = rate of transference from plasma to liver; 5 = rate of transference from liver to small intestine via bile duct; 6 = rate of conversion to bile acids in liver; 7 = rate of addition from the diet; 8 = rate of absorption from diet; 9 = faecal excretion; 10 = rate of transference into pool 1; 11 = rate of synthesis from acetate in pool 2; 12 = rate of transference from pool 2 to plasma; 13 = rate of transference from plasma to pool 3; 14 = rate of synthesis of cholesterol in pool 3; 15 = rate of transference from pool 3 to plasma (Boyd, 1975).

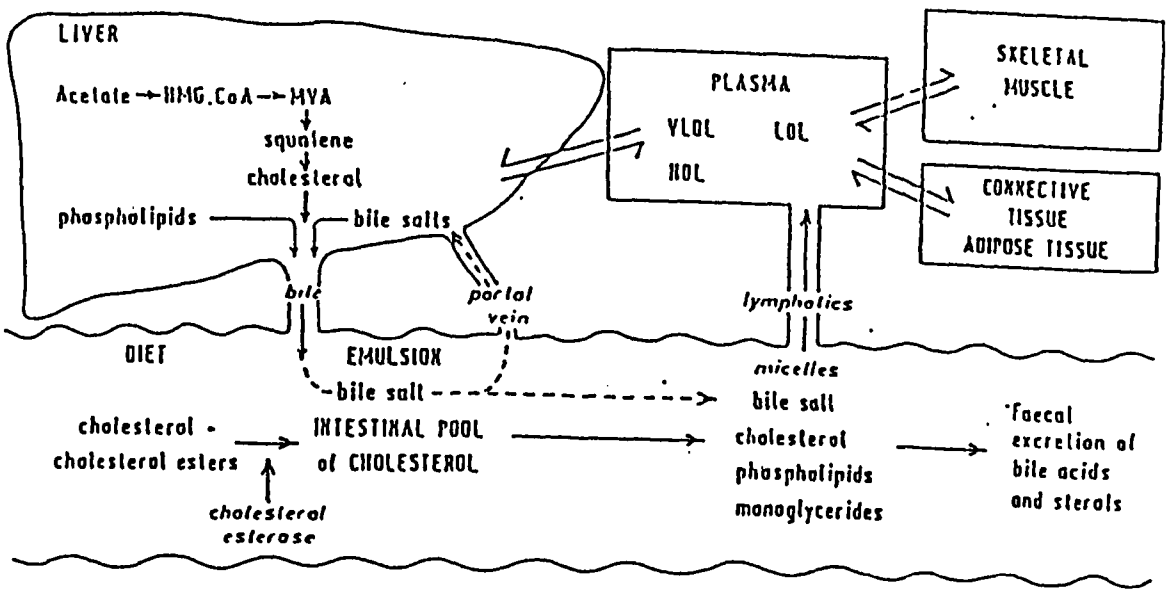

Figure 6. Interrelationship between liver, small intestine, plasma and extrahepatic tissues regarding cholesterol exchanges. MVA = Mevalonic acid, HMG CoA = 3-hydroxy-3-methylglutaryl coenzyme A, VLDL = very-low-density lipoprotein, LDL = low-density lipoprotein, high-density lipoprotein (Boyd, 1975)

such as acetate. Liver is the only known source of enzymes involved in the conversion of cholesterol to bile acids (Boyd, 1975).

Hypercholesterolemia and Atherosclerosis

Cholesterol feeding in rabbits leads to increased cholesterol and phospholipid in plasma and erythrocytes resulting in an increased cholesterol to phospholipid ratio (Kanakaraj and Singh, 1989). Hypercholesterolemia was induced by feeding guinea pigs a purified diet containing 15% lard and enriched with 0.5% cholesterol (O'Brien and Corrigan, 1988; Hadcock et al., 1991), whereas hypercholesterolemia was inhibited significantly in pigs consuming baked beans, peas and butter beans (Kingman et al., 1993). Hypercholesterolemia (mean plasma cholesterol: 15 mM) was induced in rabbits fed a chow diet enriched with a low amount (0.25%, w/w) of cholesterol (Gross et al., 1991). Hypercholesterolemia increased both free and esterified cholesterol in the entire aorta of pigs fed a hypercholesterolemic diet (Sassen et al., 1993).

In pigs, hypercholesterolemia was associated with increased transport rates of LDL-cholesteryl ester (CE) (165 mumol/h/L) and HDL-CE (78 mumol/h/l) and with an increased flux of HDL-CE to LDL (78 mumol/h/L) (Terpstra et al., 1993). Hypertriglyceridemia and enhanced hypercholesterolemia in

rabbits fed coconut oil resulted primarily from increased hepatic secretion of VLDL and a modest decrease in VLDL triglyceride clearance capacity (Van Heek and Zilversmit, 1991). Hypercholesterolemia resulted in elevation of plasma and tissue endothelin immunoreactivity and abnormal acetylcholine-evoked coronary vasoconstriction in hypercholesterolemic animals was associated with further elevation of plasma endothelin. Endothelin was an early participant and marker for endothelium dysfunction in hypercholesterolemia and atherogenesis (Lerman et al., 1993).

Hyperlipidemia can potentiate the cardiovascular responses to adrenergic stimulation and may have some relevance to the possible role of mechanism of interaction of hyperlipidemia and hypertension in atherogenesis (Kuwahara et al., 1992). In rabbits fed a high cholesterol diet, plasma cholesterol concentration elevated significantly without macroscopic evidence of atherosclerosis after 4 weeks, whereas after 8 weeks significant development of atherosclerotic lesions was present in the thoracic and abdominal aorta (Du and Woodman, 1992). High dietary cholesterol caused increased aortic intimal-plus-medial thickness (Ludwig et al., 1992). Low-level cholesterol-enriched diet (0.1%) was atherogenic, with macroscopically detectable lesions of atherosclerosis apparent at 12 weeks (Teixeria et al., 1991).

Combination of feeding a cholesterol/fat dietary regimen

and chronic mild endothelial injury in rabbits resulted in a diet-induced hypercholesterolemia with LDL as the predominant lipoprotein; and lesion within the iliac-femoral artery comparable in histologic and chemical composition to human fatty streak (Bocan et al., 1991). Short-term diet-induced hypercholesterolemia increased the size of myocardial infarcts in awake dogs (Sakamoto et al., 1991).

Atherosclerosis is a complex-multifactorial disease which results when there is formation of calcified plaque with intermediate events of intimal cell injury, smooth muscle cell proliferation and migration, macrophage infiltration, lipid accumulation and excess formation of ground substance (Nayler, 1992). Atherosclerosis is not merely a disease, but a process that is the principal contributor to the pathogenesis of myocardial and cerebral infarction, gangrene and loss of function of the extremities (Ross, 1993).

Several calcium-dependent processes are involved in atherosclerotic lesion formation; platelet aggregation, monocyte adhesion, release of growth factors, cell proliferation and migration, protein and collagen secretion and synthesis and endothelial necrosis (Nayler and Panagiotopoulos, 1993). Macrophage/smooth muscle interactions play a role in atherogenesis and foreign body reactions to biomaterials (Lam et al., 1992). Diabetes mellitus (plasma glucose greater than 16 mM) is a major risk factor for

atherosclerosis. In alloxan-induced diabetes in normally fed rabbits, endothelial alterations consistent with injury, included adhesion of white blood cells, platelets and fibrin-like material to endothelial surface of aortas after 2 weeks (Hadcock et al., 1991). Loss of endothelium relaxation is one of the early steps in atherogenesis (Becker et al., 1991). Endothelial cell damage is considered to be the primary event in atherogenesis; cholesterol levels have correlated significantly with endothelial damage of coronary arteries (Pesonen et al., 1987). Selective endothelial dysfunction giving rise to abnormal coronary artery reactivity preceded the onset of coronary artery atherosclerosis during hypercholesterolemia (Cohen et al., 1988).

Lipolytic remnants of triglyceride-rich lipoproteins may play an important role in the development of atherosclerosis by decreasing the barrier function of vascular endothelium (Henning et al., 1992). Cholesterol oxidation products (oxysterols), cholesten-3-beta, 5 alpha,6 beta-triol (Triol), may be atherogenic by altering the barrier function of vascular endothelium (Ramasamy et al., 1992).

Progression of atherosclerotic lesions in coronary arteries and abdominal aortas in swine fed hyperlipidemic diet showed lesions mainly in the intima cell masses. Lipid-rich calcific necrotic debris occupied about 25% of the lesion and lesions increased 3-fold in size and cell numbers (Kim et al., 1988).

Cholesterol accumulation in the tunica intima of arteries is a characteristic of atherosclerosis. Activation of the complement system by cholesterol producing chemotactic products for monocytes may provide a mechanism for lipid accumulation in arteries (Seifert et al., 1989). Observations in animals showed that fatty streaks precede the development of intermediate lesions (Ross, 1993). In a rabbit surgical model, higher cholesteryl ester influx led to cholesterol accumulation in arteries (Hjelms and Stender, 1992). Positive association existed between permeability and accumulation of cholesterol in the aortic segment of cholesterol-fed rabbits (Nielsen et al., 1992). Enhanced rate of development of coronary atherosclerotic during hypercholesterolemia is more critically modulated by genetic factors than by absolute plasma cholesterol concentration (Sprecher et al., 1987). Lesion development in the aorta of cholesterol-lard fed swine correlated with foam cell accumulation (Cupp et al., 1987). Atherosclerotic lesions comparable in composition to human fatty streaks were induced in the iliac-femoral artery of hypercholesterolemic New Zealand White rabbits by chronic endothelial denudation (Bocan et al., 1991). Watanabe heritable hyperlipidemic (WHHL) rabbits have elevated concentrations of plasma cholesterol and develop progressive atherosclerosis (Wines et al., 1989). Lipoprotein changes seen in male WHHL heterozygous (WHHL-Hh) rabbits during aging were

similar to those observed in human subjects. Pregnancy in WHHL-Hh rabbits, like in humans, may induce hyperlipidemia, which is opposite to effects observed in normal or homozygous WHHL rabbits (Esper et al., 1993). Atherosclerotic lesion development may be altered indirectly by the regulation of plasma cholesterol or indirectly by inhibition of acyl-CoA cholesterol O-acyltransferase within arterial cells (Bocan et al., 1993). Evidence suggests that atheroma formed in a short period in rabbits fed a cholesterol diet were caused mainly by increased LDL1, of large molecular size and foam cells formed in humans atheromas were caused by the production of modified LDL with peroxidized cholesteryl ester (Kanazawa et al., 1993).

In cholesterol-fed rabbits receiving dietary antioxidants, results suggest that abnormalities in endothelium-dependent control of vascular tone developed early in atherosclerosis and may result from oxidative modification of LDL (Keaney et al., 1993). Low-density lipoprotein oxidation and macrophage accumulation are both involved in atherogenesis (Aviram and Eias, 1993; Kita et al., 1992; Kita et al., 1990). Oxidative modification of LDL may represent an initiating event in the formation of monocyte-macrophage foam cells, a major cell type in fatty streaks and atherosclerotic fibrous plaques (Bocan et al., 1992). Oxidation of LDL in atherosclerotic may be involve in converting macrophages into cholesterol-laden foam cells

(Morgan et al., 1993).

Postsecretory modifications of lipoprotein appear to influence atherogenesis, and pro- and antioxidative conditions can modulate these processes (Mol et al., 1993; Mao et al., 1991). Platelet-derived serotonin may contribute to thromboembolic complications of atherosclerosis (Weber et al., 1993). High fat-cholesterol intake could play a causative role in matrix dysfunction by altering the synthesis of glycoprotein during atherogenesis (Sharma et al., 1993).

Susceptibility studies of animals to atherosclerosis showed that animals with high polyunsaturated to saturated fatty acid ratio (mice, 13.0, tree shrews, 9.0, rats, 9.0) were resistant to atherosclerosis, whereas animals with low polyunsaturated to saturated fatty acid ratio (rabbits, 2.9) were susceptible. Polyunsaturated to saturated fatty acid ratio of 4.9 in men, pigs, 4.2 and monkeys, 3.7, were between those of the resistant and susceptible animals (Chen and Li, 1993). Hepatic lipase plays a key role in turnover of atherogenic remnants and distribution of HDL particle size subclasses. Rabbits fed a cholesterol-enriched diet accumulated potentially atherogenic chylomicron remnants and VLDL (Ebert et al., 1993). Lipoprotein remnants play an important causal role in atherosclerosis under conditions where plasma lipoproteins levels are elevated (Nordestgaard and Lewis, 1991).

Treatment of hypercholesterolemia by diet or by drugs

decreased the incidence of coronary heart disease (Katan, 1992). Supplementation of swine diet with vitamin D_3 increased plasma cholesterol levels but decreased arterial damage (Ito et al., 1987). Dietary supplementation with evening primrose oil in New Zealand White rabbits may be effective in the prevention of atherosclerosis (Fragoso and Skinner, 1992). Supplementation of standard rabbit diet with olive oil or margarine has led to lower plasma cholesterol accumulation in the thoracic aorta (Hodis et al., 1992). Dietary magnesium prevented development of atherosclerosis in cholesterol-fed rabbits by inhibiting lipid accumulation in aortic wall (Ouchi et al., 1990). Intervenous injection of polyene phosphatidylcholine in rabbits decreased plasma cholesterol and increased HDL-cholesterol concentrations and decreased the extent of aorta damage (Tarkhovskaia et al., 1992). Thermofiltration lowering of plasma cholesterol levels resulted in slowing the progression of atherosclerosis (Noruma et al., 1989).

LDL from normal rabbits has acted as antiatherogenic lipoprotein (Kanzawa et al., 1991). The antiatherogenic properties of HDL are thought to reside in their involvement in the reverse cholesterol transport pathway (De Cromp et al., 1992). Skim milk has slowed down the process of cholesterol-induced atherogenesis (Aggarwal and Kansal, 1992). Human monocyte colony-stimulating factor enhanced excretion of

cholesterol from target cells (cholesterol-laden macrophages) in arterial walls, reducing the rate of atherogenesis (Yamada et al., 1992). Dietary fish oils has exacerbated LDL hypercholesterolemia, but not the atherosclerotic process in casein-fed rabbit model for atherosclerosis (Adelstein et al., 1992). Fish oils have attenuated atherosclerosis in cholesterol-fed rats, quails, rabbits, pigs and monkeys (Zhu and Parmlet, 1990). Dietary fish oils decreased aortic lipid deposition in WHHL rabbits, possibly by decreasing plasma triglyceride and cholesterol, platelet count and aggregability and systolic blood pressure (Lichtenstein and Chobanian, 1990). In male Yorkshire pigs fed a 2% high-cholesterol diet, cod-liver oil delayed the impairment of endothelium-dependent relaxations in hypercholesterolemia and atherosclerosis (Shimokawa and Vanhoutte, 1988). Dietary grapefruit pectin supplementation inhibited hypercholesterolemia and appeared to be protective against atherosclerosis (Baekey et al., 1988).

Unmodified estrogens decreased atherosclerosis without damaging the rabbit liver (Skjaerlund, 1992). Moderate pharmacological doses of estrogen given to cholesterol-fed rabbit for 12 weeks drastically retarded the development of arterial lesions (Henriksson et al., 1989). The antioxidant action of ferulic acid in gamma-oryzanol in hypercholesterolemic New Zealand White rabbits decreased the incorporation of oleate into cholesteryl ester by macrophages

(Hiramatsu et al., 1990). ACAT inhibitor C-976 in the absence of plasma cholesterol lowering drugs has resulted in inhibition of atherosclerosis lesion progression and can enhance regression (Bocan et al., 1991). Probucol has lowered plasma cholesterol in humans and animals. It reduced progression of atherosclerosis in LDL receptor-deficient Watanabe rabbits (Mao et al., 1991). Probucol slowed the progression of atherosclerosis, especially foam cell-rich fatty steak lesions (Kita et al., 1991). Probucol has decreased oxidative modification of LDL and diminish its uptake by macrophages (Faulkner et al., 1993).

Panax notoginseng saponins may have prevented atherosclerosis and inhibited progression of atherosclerotic lesions by interfering with proliferation of arterial smooth muscle cells (Lin et al., 1993). Abana administration in hypercholesterolemic rabbits showed pronounced reduction in atherosclerotic involvement of coronary artery (Tiwari et al., 1993). Regression of plaque was a function of dynamic balance among initiation, progression, stabilization and removal of plaque constituents (Schwartz et al., 1992).

Modifications of Cholesterol By Cholesterol Lowering Modalities

Studies have shown that the type of dietary protein can influence cholesterol metabolism. Rabbits fed casein and soy protein showed different responses. Rabbits readily developed

hypercholesterolemia when fed a casein diet, and substitution of the casein diet with a soy protein diet decreased serum cholesterol concentration (Terpstra, 1983). Injection of [26-^{14}C]cholesterol to rabbits fed casein or a soy protein showed that the decay in plasma cholesterol specific radioactivity decreased much more rapidly in rabbits fed the soy protein diet than in rabbits fed the casein diet. Increased fecal steroid excretion, increased oxidation of cholesterol in the liver and decreased secretion of cholesterol pointed to the mechanism responsible for the hypocholesterolemic effect in rabbits and rats fed soy protein compared with casein (Sugano, 1983). In cholesterol-fed rats, soybean protein diet decreased plasma cholesterol when compared with a casein diet (Chizuko et al., 1983).

Lipid-lowering drugs may have affected the kinetics and transport of cholesterol. Reverse cholesterol transport which includes transport to the liver, represents the movement of cholesterol out of cells and regulating flux of sterol through membranes. Probucol is believed to act in lowering cholesterol as a primary antioxidant, whereas neomycin is thought of as complexing bile acid in the gut similar to cholestyramine (Schroeder et al., 1991). In rabbits fed a 0.5% cholesterol-rich diet supplemented with 1% probucol, an inhibitor of LDL oxidation, prevented increase in lipid peroxides (Simon et al., 1993). Intravenous administration of cyclodextrins to

rats decreased plasma cholesterol level in a dose-dependent manner (Frijlink et al., 1991). Lifibrol modified lipid metabolism in atherosclerotic aortae from swine and WHHL rabbits during incubation has implications to extend its ability beyond its confirmed plasma cholesterol-lowering activity (Bell, 1993). Etofibrate treatment of rabbits fed a 0.1% cholesterol diet markedly decreased total cholesterol and LDL-cholesterol and decreased copper-induced lipid peroxide formation (Wulfroth et al., 1992).

In cholesterol-fed rabbits, lovastatin, a HMG CoA reductase inhibitor, the observed hypocholesterolemic effect was associated with decreased cholesterol absorption from the intestinal tract (Nielsen et al., 1993). In New Zealand White rabbits fed a cholesterol diet with pravastatin and simvastatin, plasma cholesterol decreased by 55%, liver membrane cholesterol by 29% and bile cholesterol saturation by 23%, liver and mononuclear cell LDL receptor activities increased by 120% and 77%, respectively in pravastatin-treated rabbits. In the simvastatin-treated rabbits, plasma cholesterol decreased by 74%, liver membrane cholesterol by 24% and bile cholesterol saturation by 38%, liver and mononuclear cell LDL receptor activities increased by 80% and 62%, respectively (Roach et al., 1993).

In normocholesterolemic rabbits fed food supplemented with 20 mg/kg body weight of pravastain, lovastatin, simvastatin

and fluvastatin for two weeks showed no modification in rabbit plasma cholesterol concentrations (Soma et al., 1993). Lovastatin and simvastatin lowered levels of plasma cholesterol in rats, dogs and rabbits by inhibition of cholesterol synthesis and induction of LDL receptor in liver cells (Choa et al., 1991). Administration of CS-514, an inhibitor of HMG CoA reductase, with and without cholestyramine to heterozygous WHHL rabbits, showed that CS-514 alone lowered plasma LDL cholesterol by 50%, and in combination with cholestyramine, lowered plasma LDL by 80% (Kume et al., 1989).

In testosterone-treated, cholesterol-fed, castrated male rabbits, aortic cholesterol content was lower than in the placebo group (Larsen et al., 1993). Intravenous injections of recombinant human monocyte colony-stimulating factor (rh-MCSF) in LDL receptor-deficient WHHL rabbits showed decreased plasma total cholesterol levels with decreased lipoproteins containing apo B 100, VLDL, intermediate-density lipoprotein (IDL), and LDL (Shimano et al., 1990). Acarbose, a complex oligosaccharide of microbial origin, added to an atherogenic diet and fed to New Zealand White rabbits decreased plasma cholesterol, IDL, and LDL (Kritchevsky et al., 1990).

Ethylenediamine tetraacetic acid (EDTA) supplemented at 3 g/day to a 0.1% cholesterol diet fed to New Zealand White rabbits lowered hepatic cholesterol concentrations (Uhl et

al., 1992). Saponins inhibited cholesterol absorption and decreased plasma cholesterol levels in experimental animals (Long et al., 1993). In hypercholesterolemic rabbits extracorporeal circuits containing enzymes modified lipoproteins and decreased plasma cholesterol concentration (Shefer et al., 1993; Labeque et al., 1993).

Cholesterol and its esters are associated with milk fat globules (Long et al., 1990), and have been found in free fat, bound to lipoprotein of fat globules and in cholesterol esters (Lacroix et al., 1973). Cholesterol in milk may be derived from blood or from de novo synthesis from acetate in the mammary gland (Long et al., 1990). The effect of dietary cholesterol on cardiovascular disease and atherosclerosis is still a great health concern and probably has contributed to the reduction in milkfat consumption (Xiansheng et al., 1990).

Food with significant levels of lipid oxidation or lipid degradation products can be defined as 'problem food'. Research on food analysis, absorption by humans and toxicology is needed (Addis and Warner, 1992). Investigators have been concerned about the conditions that oxidize cholesterol and the effect that the oxidized products have on diet and health (Cleveland and Harris, 1987). Informed consumers prefer low fat products (Schroder and Baer, 1990; Xiansheng et al., 1990), and product manufacturers need more applicable inexpensive methods to decrease excess cholesterol and fat in

foods.

Numerous chemical and physical methods have been employed to reduce or eliminate cholesterol in milk. The use of organic solvents results in residues in the final product. Supercritical fluid extraction, vacuum steam distillation, short path distillation, and complexing with cyclodextrins and saponins are used also (Kosikowski, 1990; Schroder and Baer, 1990; Xiansheng et al., 1990; Micich, 1991; Smith et al., 1991). Enzyme-based processes have been employed in the food industry (Gross, 1991; Kosikowski, 1988).

The chemical/physical methods used to reduce or eliminate cholesterol from foods are expensive and non-selective removing flavor and nutrient components along with cholesterol (Smith et al., 1991). Enzymatic processes have major selective advantages: highly selective, operation near room temperature, reduce by-product formation and potentially minimize regulatory hurdles (Gross, 1991; Smith et al., 1991).

Studies have suggested that unsaturated dietary sterols were hydrogenated by rumen microflora; this was confirmed when cholesterol was hydrogenated to coprostanol during anaerobic incubation with rumen fluid. Microorganisms capable of reducing cholesterol in the intestinal tract are of the genera *Bifidobacterium, Clostridium* and *Bacteriodes*. Microorganisms that hydrogenate cholesterol to coprostanol have been isolated from rat ceca, human and baboon feces. They are small, gram-

positive, strictly anaerobic and are assigned tentatively to the genus *Eubacterium* (Dehal et al., 1991). *Lactobacillus acidophilus* RP32 significantly inhibited increase in serum cholesterol levels in pigs fed a high-cholesterol diet (Gilliland et al., 1984). *Eubacterium 21,408* reduced the 4,5 double band of allocholesterol to coprostanol (Eyssen et al., 1973). Hydrogenation of cholesterol by anaerobic incubation with rumen fluid for 20 hours resulted in coprostanol as the principal product (Ashes et al., 1978). Results of catalytic hydrogenation of cholesterol indicated that coprostanol was one of the by-products (Hershberg et al., 1951).

Streptococcus faecalis KAWI decreased serum cholesterol levels in humans with hyperlipidemia as a result of decreased LDL-cholesterol. The mechanism of serum lipid reduction in rabbits and rats showed that serum cholesterol level was decreased by oral administration of *Streptococcus faecalis KAWI* (Suegara et al., 1985). *Escherichia coli* in human feces was found to degrade cholesterol under aerobic and anaerobic conditions to form neutral products, cholest-4 en-3-one, cholesta-1,4-dien-3-one, androst-4-ene-3,17-dione, and androsta-1,4-diene-3,17-dione. Cholesta-1,4-dien-3-one and androsta-1,4-diene-3,17-dione were also produced under anaerobic condition (Owen et al., 1978). Degradation of cholesterol by *Pseudomonas species NCIB 10590* showed that the major products included cholest-5-en-3-one, cholest-4-en-3-

one, 26-hydroxycholest-4-en-3-one, androst-1,4-dien-3,17-dione, cholest-4-en-3-one-26-oic acid, chol-4-en-3-one-24-oic acid, pregn-4-en-3-one-20-carboxylic acid and pregna-1,4-dien-3-one-20 carboxylic acid (Owen et al., 1983). Microbial hydrogenation of cholesterol to coprostanol may occur by the intermediates 4-cholesten-3-one and coprostanone (Eyssen et al., 1973). Microbial transformation of cholesterol by cecal contents from rats showed that at least 50% of the cholesterol converted to coprostanol by means of the intermediate 4-cholestenone (Bjorkhem and Gustafsson, 1971). In the human gut, cholesterol is partially degraded to coprostanol and coprostanone and determination of metabolites is complicated by the degradation of products from plant sterols (Macdonald et al., 1983). *Mycobacterium* species were magnetically immobilized and used for side chain degradation of cholesterol into androsta-1,4-dien-3,17-dione (Flygare and Larsson, 1978).

Demonstrations have shown that bacteria as well as plants can metabolize steroids by transformation of their side chains and nucleus to produce pharmaceutically active compounds. Dehal et al. (1988) showed that cytosolic preparations from cucumber leaves contained cholesterol reductase activity (Dehal et al., 1989). Further demonstrations showed that cholesterol reductase activity was present in alfalfa and garden pea leaves (Dehal et al., 1991). Fluorometric assay of cholesterol reductase activity in peas leaves (*Pisum sativum*)

showed when cholesterol is catalytically reduced by cholesterol reductase oxidation of NADH occurred (Yang and Beitz, 1992). Results suggested that fluorometric method was useful for the accurate determination of cholesterol reductase activity in biological specimens.

Freier (1991) successfully isolated a pure culture of *Eubacterium coprostanoligenes* and it was characterized as a small, gram positive, non-spore forming, coccobacillus, and cholesterol-reducing anaerobe. Lecithin was required for growth, whereas cholesterol was not. Coprostanol is not produced when lecithin is replaced by phosphatidylinositol or phosphatidylglycerol. *E. coprostanoligenes* possesses phospholipase activity and does not reduce nitrate or produce indole. Esculin was hydrolyzed, but starch and gelatin were not. *E. coprostanoligenes* produced much acid by fermentation of amygdalin, lactose and salicin (acetic, formic and succinic), whereas arabinose, cellobiose, fructose, glucose, mannose and melibiose were poorly fermented. Volumes of gases produced in the head space of fermentation apparatus showed that H_2 and CO_2 percentages ranged from 4.5 to 7.2 and 0.9 and 1.8, respectively. The evaluation of the effects of pH on coprostanol production by *E. coprostanoligenes* showed that the greatest amounts of coprostanol was formed in media with pH values 7.5, 7.2 and 7.0. After incubation, the pH values of the media used were 6.4, 6.3, and 6.2, respectively (Freier et

al., 1994).

Fermentation of radiolabelled [4-^{3}H, 4-^{14}C]-cholesterol with *E. coprostanoligenes* resulted in 90% conversion of cholesterol to coprostanol in 5 days. Coprostanol formation by *E. coprostanoligenes* involved the transfer of a hydrogen from C4 to C5 position. *E. coprostanoligenes* reduction of radioactive coprostanone to coprostanol tend to favor the theory of the indirect pathway with the isomerization of a double bond at the 4-5 position (Ren, 1991).

Oral administration of *E. coprostanoligenes* to New Zealand White rabbits fed a 0.05% cholesterol-enriched diet resulted in lower ($P < 0.001$) plasma cholesterol concentration in rabbits fed the live bacteria for 10 days when compared with rabbits fed the boiled bacteria. Coprostanol/cholesterol ratios in the contents of the digestive tracts of rabbits fed the live bacteria were greater than those of rabbits fed the boiled bacteria (Li et al., 1995)

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USE OF MILK TO DELIVER *EUBACTERIUM COPROSTANOLIGENES* TO DECREASE PLASMA CHOLESTEROL CONCENTRATION IN HYPERCHOLESTEROLEMIC RABBITS

A paper to be submitted to the Journal of Lipid Research

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Abstract

The use of milk to deliver *Eubacterium coprostanoligenes*, a cholesterol-reducing bacteria, to decrease plasma cholesterol concentration was investigated in hypercholesterolemic rabbits. Eighteen adult male New Zealand White rabbits were randomly divided into three groups and fed a no cholesterol diet (control), control diet supplemented with 0.1% cholesterol and boiled *E. coprostanoligenes* in 2% minimal fat homogenized milk diet or control diet supplemented with 0.1% cholesterol and *E. coprostanoligenes* in 2% fat homogenized milk for 21 days.

No clinical signs or symptoms of bacterial intoxication were observed in any rabbit. There were no significant differences in rabbit mean body weight during the different feeding periods. There were no significant differences between total plasma cholesterol concentration in rabbits fed the 0.1% cholesterol + boiled *E. coprostanoligenes* in 2% fat

homogenized milk diet, and a 0.1% cholesterol + *E. coprostanoligenes* in 2% fat homogenized milk. However, changes in total plasma cholesterol concentration from day 0-77 in rabbits fed the 0.1% cholesterol + *E. coprostanoligenes* in 2% fat homogenized milk diet were significantly lower ($P < 0.01$) than plasma cholesterol concentration in rabbits fed the 0.1% cholesterol + boiled *E. coprostanoligenes* in 2% fat homogenized milk diet. Liver enzymes activities and major metabolites in plasma were not influenced by the bacterial treatments.

Fecal excretion of cholesterol, coprostanol, sitosterol and bile acid in rabbits fed the 0.1% cholesterol + *E. coprostanoligenes* in 2% fat homogenized milk diet were greater at day 77 compared with that excreted by rabbits fed the 0.1% cholesterol + boiled *E. coprostanoligenes* in 2% fat homogenized milk diet. Fecal bile acid excretion was 1.9 times greater ($P < 0.05$) in rabbits fed the 0.1% cholesterol + live *E. coprostanoligenes* in 2% fat homogenized milk diet.

Fecal coprostanol/cholesterol ratio of rabbits fed the 0.1% cholesterol + live *E. coprostanoligenes* in 2% fat homogenized milk diet at day 77 was greater 1.7 times greater than that of rabbits fed the 0.1% cholesterol + boiled *E. coprostanoligenes* in 2% fat homogenized milk diet. In summary oral administration of *E. coprostanoligenes* via milk decreased plasma cholesterol conversion of intestinal cholesterol to

coprostanol and increased excretion of cholesterol, coprostanol and bile acids. Therefore, milk may be used as food to deliver a hypocholesterolemic microbe to people. (Supported in part by Anderson Erickson Dairy Company, Iowa Dairy Products Association, and the Center for Redesigning Foods to Improve Nutrition).

INTRODUCTION

Studies have demonstrated a causal association between serum cholesterol concentration, dietary fat, and coronary heart disease, and it is believed that the risk of coronary heart disease should be reduced substantially when serum cholesterol concentration is lowered (1). Cholesterol content of animal-derived foods is a primary public concern as there is considerable amount of evidence that suggest that plasma cholesterol plays an important role in the development of atherosclerosis and coronary heart disease.

Cholesterol is required for several important biological functions in animals, including humans; precursor for biosynthesis of bile acids, corticosteroids, sex hormones and structure and function of membranes (2).

Recent investigations have shown that stearic acid failed to increase LDL-cholesterol concentration in plasma, whereas oleic acid was as effective as linoleic acid in lowering

plasma LDL-cholesterol when substituted for palmitic acid (3). In a study conducted on the output of bile and biliary components (biliary salts, phospholipids and cholesterol) during the first hour after bile duct catheterization in rats, it was found that feeding a commercial diet or semi-purified diets containing 7 or 20 parts/100 of corn oil or 20 parts/100 of lard or mutton tallow, the output of cholesterol was augmented when the percentage of corn oil was increased from 7 to 20 parts/100 (4).

In studies conducted on the effect of a bean diet on biliary lipid secretion, serum cholesterol concentration, and hepatic cholesterol metabolism in rats, the results showed increased output of biliary cholesterol and biliary phospholipid, whereas biliary flow and biliary salts output remained in the normal range. Total serum and very-low-density lipoprotein (VLDL) cholesterol concentrations decreased significantly (27% and 50%), whereas hepatic cholesterogenesis increased 170% in the bean fed animals. Newly synthesized cholesterol contribution to total biliary cholesterol increased by 200% and was preferentially channelled to the biliary cholesterol secretion in these rats (5).

Lipid lowering drugs may affect the kinetics and transport of cholesterol. Probucol is concluded to act in lowering cholesterol as a primary antioxidant, whereas neomycin is thought to complex bile acids in the gut in a manner similar

to cholestyramine (6) In a study of the relationship between rates of hepatic sterol synthesis and rates of hepatic low-density lipoprotein (LDL) uptake (clearance) in animals with high (rats), low (female hamsters) and very low (male hamsters) basal rates of hepatic sterol synthesis, it was found that in rats and female hamsters fed cholesterol or cholestyramine, rates of hepatic sterol synthesis varied over 110-fold range, whereas rates of hepatic LDL clearance and plasma LDL-cholesterol concentrations remained unchanged. In male hamsters cholestyramine feeding increased rates of hepatic LDL uptake by 2.5-fold and was associated with a 50% reduction in plasma LDL-cholesterol concentrations (7).

Swine have been used as model in several research studies related to human problems (diseases) because of their similarities to human in cardiac and vascular anatomy, ventricular performance, electrophysiology and coronary artery distribution which should result in transition from research to clinical application (8). The nutrient requirements of the pig resembles that of human more than any other nonprimate mammalian species due the physiological and anatomical similarity of their digestive tract which provide the basis for use in human nutritional studies (9).

In swine fed soy and beef diets, although there was no significant difference in plasma cholesterol concentrations, there was increased proportion of fat in carcass and increased

cholesterol deposition in tissue (10). In hypercholesterolemic pig fed baked beans at 300 g/kg diet, plasma cholesterol decreased by 36.5%, whereas low-density-lipoprotein-cholesterol decreased by 48% (11).

In ruminants, several studies suggested that unsaturated dietary sterols were hydrogenated by rumen microflora. This suggestion was confirmed when cholesterol was hydrogenated to coprostanol during anaerobic incubation with sheep rumen fluid for 20 hours (12). Several studies have suggested that dietary administered microorganisms influenced plasma cholesterol concentration. *Lactobacillus acidophilus RP32* significantly inhibited increase in serum cholesterol in pigs fed high-cholesterol diet (13).

Demonstrations have shown that bacteria as well as plants can metabolize steroids by transformation of their side chains and steroid nuclei to produce pharmaceutically active steroid compounds. Dehal et al. (1988) showed that cytosolic preparations from cucumber leaves contained cholesterol reductase activity (1). Further demonstrations showed that cholesterol reductase activity was also present in alfalfa and in garden pea leaves (17).

Eubacterium 21,408 reduced the 4,5 double band of allocholesterol to coprostanol (14). It was found that *Streptococcus faecalis KAWI* decreased serum cholesterol levels in human with hyperlipidemia and serum cholesterol levels in

rabbits and rats by oral administration (15). Microbial transformation of cholesterol by cecal contents from rats showed that at least 50% of the cholesterol was converted to coprostanol (16). The conversion of cholesterol to coprostanol was observed also in rats after intervenous injections of an alfalfa cholesterol reductase (1).

Freier (1991) successfully isolated a pure culture of *E. coprostanoligenes* and it was characterized as a small, gram positive, nonspore forming, coccobacillus and cholesterol-reducing anaerobe. Lecithin is required for growth, whereas cholesterol is not. Coprostanol is not produced when lecithin is replaced by phosphatidylinositol or phosphatidylglycerol. *E. coprostanoligenes* possesses phospholipase activity and does not reduce nitrate or produce indole. Esculin is hydrolyzed, but starch and gelatin is not. *E. coprostanoligenes* produces acids (acetic, formic and succinic) by fermentation of amygdalin, lactose and salicin, whereas arabinose, cellobiose, fructose, glucose, mannose and melibiose are poorly fermented. Percentages of H_2 and CO_2 in the head space during growth ranged from 4.5 to 7.2 and 0.9 to 1.8, respectively. Evaluation of the effects of pH on coprostanol production by *E. coprostanoligenes* showed that greater amounts of coprostanol were formed in media with pH values of 7.5, 7.2, and 7.0. After fermentation pH values of the media used were 6.4, 6.3, and 6.2, respectively (18,19).

Fermentation of radiolabelled [4-^{3}H, 4-^{14}C]-cholesterol with *E. coprostanoligenes* resulted in 90% conversion of cholesterol to coprostanol in 5 days. Coprostanol formation by *E. coprostanoligenes* involved the transfer of a hydrogen from C4 to C5 position. *E. coprostanoligenes* reduction of radioactive coprostanone to coprostanol tend to favor the theory of the indirect pathway with the isomerization of a double bond at the 4-5 position (20).

Oral administration of *E. coprostanoligenes* to New Zealand White rabbits fed a 0.05% cholesterol-enriched diet resulted in lower ($P < 0.001$) plasma cholesterol concentration in rabbits fed the live bacteria for 10 days when compared with rabbits fed the boiled bacteria. Coprostanol/cholesterol ratios in the contents of the digestive tracts of rabbits fed the live bacteria were greater than those of rabbits fed the boiled bacteria (21).

The study was undertaken to investigate the ability of *E. coprostanoligenes* to decrease plasma cholesterol concentration in hypercholesterolemic rabbit as a model for human hypercholesterolemia. The hypothesis of the study is that orally administered *E. coprostanoligenes* will convert dietary and endogenous cholesterol in the small intestine to coprostanol which is a poorly absorbed sterol (2), and increased excretion of total fecal steroids. It is further hypothesized that increased excretion of fecal steroids will

decrease plasma cholesterol concentrations.

The specific objectives of the study were; to feed a cholesterol-enriched diet to make the rabbits hypercholesterolemic and to evaluate the effects of dietary administered *Eubacterium coprostanoligenes* on (a) rabbit plasma cholesterol concentration (b) rabbit health and c) fecal coprostanol to cholesterol ratios.

MATERIALS AND METHODS

Animals and diets

Eighteen (12 weeks old, 3 kg body weight) New Zealand White male adult rabbits (Laboratory Animal Resources, College of Veterinary Medicine, Iowa State University) were used in this experiment. The rabbits were housed and fed separately. Fourteen rabbits were randomly selected and fed a commercial chow with cholesterol (Sigma Chemical Co., St. Louis, MO) added at 0.1% on dry-matter basis. The remaining 4 rabbits were fed the commercial chow without cholesterol (control: Group 1). Plasma cholesterol was monitored weekly in the cholesterol-fed rabbits until hypercholesterolemia was maintained. The rabbits were then randomly assigned to two treatment groups as follows: Group 2 = Cholesterol + boiled *E. coprostanoligenes* in 2% fat milk, N = 6 and group 3 = Cholesterol + *E. coprostanoligenes* in 2% fat milk, N = 8.

Rabbits were maintained on the commercial chow or on the 0.1% cholesterol-enriched diet for 21 days before feeding the *E. coprostanoligenes*-containing diets.

Preparation of bacterial culture

E. coprostanoligenes ATCC 5122 was grown anaerobically in liquid media composed of 0.2% cholesterol, 0.1% lecithin, 0.5% sodium thioglycollate (Sigma Chemical Co., St. Louis, MO), 1% casitone, 1% yeast extract (Difco Laboratories, Detroit, MI), and 0.1% calcium chloride (Fisher Scientific, Fair Lawn, NJ). Resazurin was added at 0.4% as an indicator for the presence of oxygen. The components were blended and the pH of the media adjusted to 7.2. The media were transferred to 400 ml boiling flasks and boiled for 30 minutes under a steady stream of argon gas until the color changed to yellow. The flasks were cooled to room temperature in an ice-bath, stoppered, then autoclaved at 121° C for 45 minutes.

Each flask was inoculated with a 4 ml aliquot of a 48 hour anaerobically-grown stock bacterial culture by using the Hungate technique (22) and incubated at 37° C for 48 hours. A 1 ml aliquot of each culture was removed from each flask for bacterial protein determination by using the Bradford method (23). The bacterial pellets then were harvested in preweighed 200 ml centrifuge bottles in a Sorvall^{R} RC2-B refrigerated centrifuge at 7,500 rpm for 10 minutes. The weights of the

bacterial pellets were calculated for the experimental feeding. A 2% homogenized lowfat milk (Anderson Erickson Dairy Co.) was used to deliver a 5 ml dose containing of 1.64 g of *Eubacterium coprostanoligenes* to the rabbits once daily. Water was provided ad libitum.

Experimental protocol

The test diets were fed for 21 days. Blood, fecal samples and body weights were taken weekly. Bacterial feeding was initiated on day 28 and ended on day 49. Feeding of the 0.1% cholesterol-enriched diet was continued without the bacteria for 28 days to observe if there was any carryover effect of feeding the bacteria. The regular commercial chow was then fed for 31 days followed by the 0.1% cholesterol-enriched diet for an additional 29 days. The rabbits were examined daily for clinical signs and symptoms of bacterial intoxication, including anorexia, lethargy and diarrhea. The feeding regimen used in the experiment is shown in Table 1.

Analysis of blood samples

Blood samples were collected (using 6 ml syringes via the central ear artery) into polystyrene tubes containing EDTA as anticoagulant. All samples were centrifuged in a Sorvall^{R} RC2-B refrigerated centrifuge at 1,500 rpm for 10 minutes. Plasma was transferred using individual pasteur pipet to individual 2

Table 1. Diets fed to each group of rabbits during different feeding periods of the experiment

Periods (days)	Diets		
	Group 1	Group 2	Group 3
0-28	No cholesterol	0.1% cholesterol	0.1% cholesterol
28-49	No cholesterol	0.1% cholesterol + boiled bacteria in 2% fat milk	0.1% cholesterol live bacteria in 2% fat milk
49-77	No cholesterol	0.1% cholesterol	0.1% cholesterol
77-108	No cholesterol	No cholesterol	No cholesterol
108-137	No cholesterol	0.1% cholesterol	0.1% cholesterol

ml tubes and stored at -20° C until analysis of cholesterol concentrations, liver enzyme activities and major metabolite concentrations. Plasma cholesterol was quantified by an enzymatic method (Cholesterol 100, Sigma Chemical Co., St. Louis, MO). Enzyme activities (glutamic oxaloacetic transaminase (GOT), gamma pyruvic transaminase (GPT), lactic dehydrogenase (LDH) and alkaline phosphatase (ALP)) and major metabolites (bilirubin, uric acid and total protein) concentrations were determined (Roche Chemicals, Kansas, MO).

Analysis of fecal cholesterol, coprostanol and bile acids

Fecal samples collected at the time of blood collection were frozen at -80° C until analysis of excretory coprostanol/cholesterol concentration ratios. Each fecal

sample was crushed with mortar and pestle then homogenized. Duplicate 0.5 grams of each sample were weighed into corresponding labelled 20 ml extraction tubes. Extraction of cholesterol and coprostanol from each sample was performed by the addition of 2 ml of methanol-benzene (4:1, v/v) and 200 µl of acetylchloride to each sample tube and capped tightly. All tubes were then placed on a heatblock set at 100° C +/- 3° C for 1 hour, then tubes were removed from the heatblock and allowed to cool to room temperature. Five ml of a 6% K_2CO_3 solution and 2 ml of benzene were added to each sample tube which then was vortexed at for 60 seconds. All tubes were centrifuged at 2,000 rpm for 20 minutes. The top (benzene) layer of each sample extract was transferred quantitatively with individual pasteur pipet to a silanized vial (24). All samples were concentrated to dryness under a stream of nitrogen gas in a heated block at set at 65° C. All samples were flushed with nitrogen gas, capped and stored at -20° C until gas chromatographic (GC) analysis was performed.

Each sample extract was dissolved in 200 µl of chloroform and vortexed for 30 seconds. Twenty µl of each sample extract was transferred to a new silanized vial and 1 µg of cholestane added as internal standard to one of the duplicates of each sample extract. Then, all samples were concentrated to dryness in a pre-heated block at 65° C under nitrogen gas. A standard mixture of 1.0 µg of cholestane, coprostanol, cholesterol and

4-cholesten-3-one in chloroform, and another mixture of ß-sitosterol, stigmasterol, cholic acid, lithocholic acid, deoxycholic acid and chenodeoxycholic acid were prepared with and without 20 µl of a control rabbit sample extract.

Each sample was derivatized to trimethylsilyl ethers by the addition of 100 µl of 1,3 bis-trimethylsilyl trifluroacetamide in 1% trimethylchlorosilane and 100 µl of silylation grade acetonitrile (Pierce, Rockford, IL) and vortexed for 15 seconds. All capped vials were return to the pre-heated block at 65° C and allowed to derivatize for 1 hour. Each sample extract was transferred quantitatively to 200 µl inserts in vials for GC analysis (25,26,27). Each derivatized sample was in 200 µl volume and an aliquot of 10 µl of each sample extract was injected into the GC for analysis of concentrations of cholesterol and metabolites.

Analysis of cholesterol, coprostanol and bile acid concentrations in the fecal samples was done on a Hewlett-Packard 5830A gas chromatograph equipped with a flame ionization detector. Column temperature was set at 280° C, injector at 290° C, detector at 290° C and maximal oven temperature at 300° C. Separation of cholesterol and coprostanol and bile acid as trimethylsilyl ether derivatives occurred on a 0.9 M, 0.64 mm ID glass column packed with 3% SP-2250 on 100/120 Supelcoport (Supelco^{R}, Bellfonte, PA). Carrier gas was nitrogen with flow rate at 60 ml/min, air at

240 ml/min and hydrogen at 60 ml/min. The detector was equipped with an integrator and slope sensitivity was set 0.5 and paper speed at 0.7 cm/min.

Quantitation of cholesterol, coprostanol and bile acid was done by calculating the peak area of each metabolite in each sample extract corresponding to the retention time of each standard in the control sample extract and comparing the ratio of peak area of each metabolite in each sample to the respective standards in the control sample.

Statistical analysis

The data were analyzed by using the SAS System software with application of the Repeated Measures Analysis and utilizing Analysis of Variance and General Linear Model procedures. Means showing significant differences were indicated by using Duncan's Multiple Range Test. Significance was based on probability value at $P< 0.05$. Body weights were analyzed at different periods of the experiment corresponding to the feeding regimen. Plasma data were analyzed for total plasma and change in plasma cholesterol concentrations at different periods and days of the experiment. Plasma liver enzyme activities and major metabolites and fecal cholesterol, coprostanol, bile acids and ß-sitosterol concentrations were analyzed at specific days of the experiment.

RESULTS AND DISCUSSION

All rabbits were fed first with a commercial chow at 140 g/day before the start of the feeding of the 0.1% cholesterol-enriched diet. Each treated rabbit received an oral daily 5 ml volume dose of 2% homogenized lowfat milk with 1.64 g bacterial pellet containing 154.99 mg bacterial protein/ml. There were no clinical signs or symptoms suggestive of effects of the cholesterol-reducing bacteria observed in any rabbit during the experiment.

No significant differences were observed in mean body weight in rabbits at the start of the experiment ($P< 0.05$) as all animals received the same commercial chow ration prior to the start of the study. Results of body weight observed in rabbits during the different feeding periods of the experiment are shown in Table 2. Rabbits in the group 1 fed the no cholesterol diet (control) had higher mean body weight than the rabbits in the group 2 fed the 0.1% cholesterol + boiled *E. coprostanoligenes* and group 3 0.1% cholesterol + live *E. coprostanoligenes* diets, but no significant differences were observed in rabbit mean body weight for any period during experiment ($P> 0.05$). Mean body weight in control rabbits between days 108-137 was higher than that of the treated rabbits fed the 0.1% cholesterol-enriched diet but were not significantly different ($P< 0.05$)(Table 2).

Table 2. Body weight of rabbits fed a commercial chow followed by a 0.1% cholesterol-enriched diet and *Eubacterium coprostanoligenes* in 2% fat milk for 21 days.

Body Weight[a] (kg)				
	Periods of Experiment (days)			
Groups	0-28	28-49	49-77	108-137
1	3.9[b] ±.3	4.0[b] ±.3	4.0[b] ±.3	4.2[b] ±.3
2	3.8[b] ±.2	3.9[b] ±.2	3.8[b] ±.3	3.7[c] ±.3
3	3.8[b] ±.2	3.8[b] ±.2	3.8[b] ±.3	3.8[c] ±.2
Significance Level				
TRT	.458	.264	.355	.069
DAY	.247	.006	.005	.0001
TRT*DAY	.0003	.525	.625	.568

[a]Values represent means and standard errors of body weight per group of rabbits corresponding to days of experiment.
[b-c]Means in the same column with the same superscript are not significantly different ($P < 0.05$)
Bacterial feeding was initiated on day 28 and terminated on day 49. Feeding of the 0.1% cholesterol-enriched diet was continued until day 77. Regular chow was fed from day 78 to day 108, which was then followed by 0.1% cholesterol diet from day 108 to day 137.
Groups: 1 = No cholesterol (control), N = 4; 2 = 0.1% cholesterol + boiled *E. coprostanoligenes* in 2% fat milk, N = 6; 3 = 0.1% cholesterol + *E. coprostanoligenes* in 2% fat milk, N = 8.

The results of analysis of total plasma cholesterol concentrations are shown in Table 3. The mean total plasma cholesterol concentration of rabbits in group 3 fed the live bacteria (130.0 ± 11 SE mg/dl) was similar to that of rabbits in group 2 fed the boiled bacteria (130.9 ± 13 SE mg/dl) at the beginning of the bacterial feeding (day 28). Lower total plasma cholesterol concentrations were observed in rabbits in group 3 fed the live *E. coprostanoligenes* (158.5 ± 13.9 SE mg/dl) at day 35 (7 days after initiation of the feeding) when compared with that in rabbits in group 3 fed the boiled *E. coprostanoligenes* (171.3 ± 12.5 SE mg/dl)(Table 3) (Figure 1), but were not different ($P < 0.05$).

Changes in plasma cholesterol concentrations at day 7 of the bacterial feeding in rabbits were different at $P< 0.13$ (Table 4). The greatest differences in plasma cholesterol concentrations were observed at days 49 and 56 and day 77 (Table 3). Differences in total plasma cholesterol concentrations were observed through out the bacterial feeding (day 28-49) but were not statistically significant ($P< 0.05$) (Table 3). No significant differences ($P< 0.05$) were observed in total plasma and changes in plasma cholesterol concentration in rabbits group 3 fed the live *E. coprostanoligenes* in the 2% fat milk and in rabbits in group 2 fed the boiled *E. coprostanoligenes* in the 2% fat milk during periods days 0-28, 35-49, 56-77 (Table 3 and Table 4).

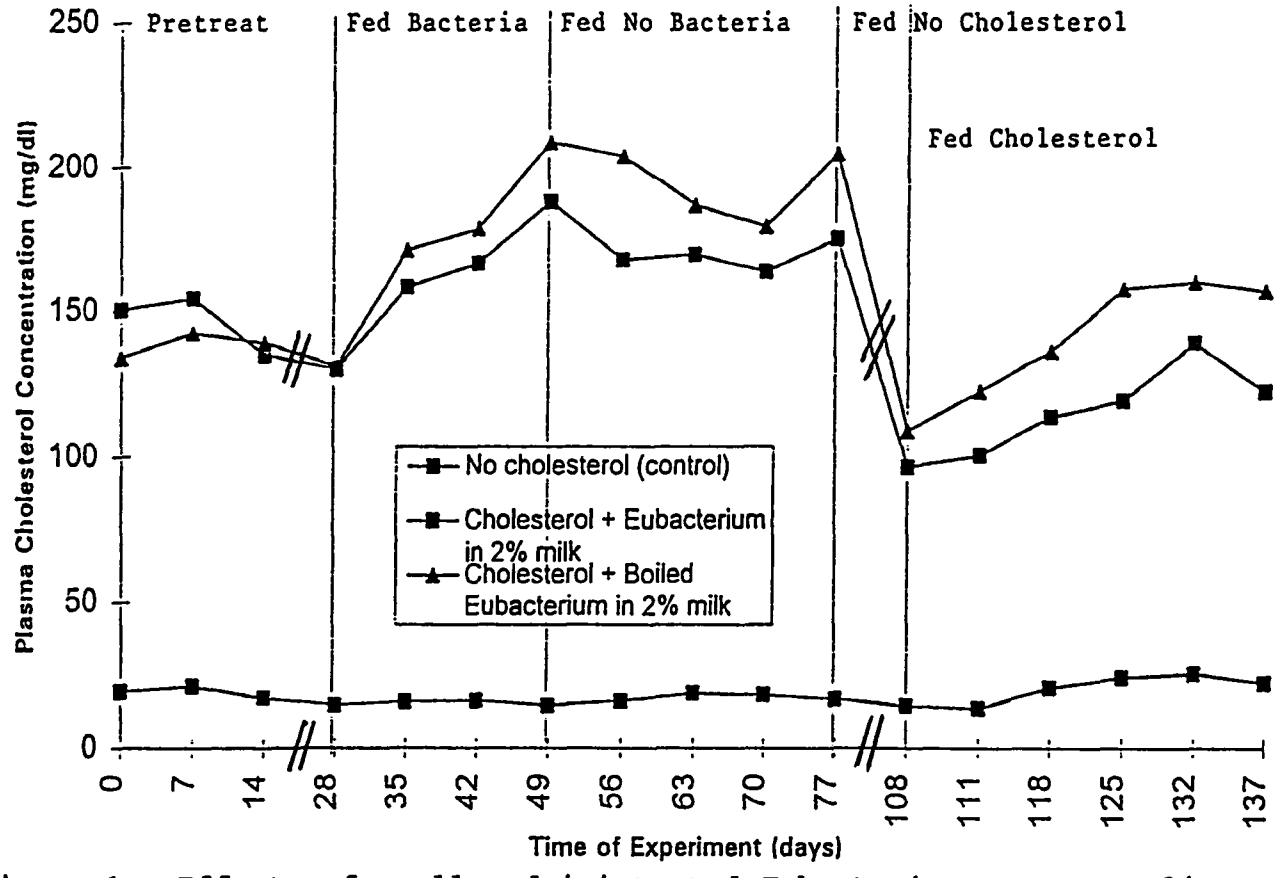

Figure 1. Effects of orally administrated *Eubacterium coprostanoligenes* on plasma cholesterol concentrations in hypercholesterolemic rabbits. All rabbits except control received a 0.1% cholesterol-enriched diet before being assigned to each group at day 0. A 2% fat milk (5 ml) was used to deliver the bacteria. Each data point represents the group average of triplicate assays of plasma samples from each rabbit; Group 1 = No cholesterol (control), N = 4; group 2 = Cholesterol + boiled *Eubacterium* in 2% milk, N = 6; group 3 = Cholesterol + *Eubacterium* in 2% milk, N = 8. *Eubacterium* was fed from day 28-49. The 0.1% cholesterol-enriched diet was fed until day 77, regular chow, day 78-108, and 0.1% cholesterol-enriched diet, day 109-137.

Table 3. Plasma cholesterol concentrations of rabbits fed a commercial chow followed by a 0.1% cholesterol-enriched diet and *Eubacterium coprostanoligenes* in 2% fat milk for 21 days

Total Plasma Cholesterol Concentrations[a] (mg/100 ml)											
	Days of Experiment										
Groups	0	7	14	28	35	42	49	56	63	70	77
1	20^{b} ±2	21^{b} ±2	17^{b} ±1	16^{b} ±2	16^{b} ±1	17^{b} ±.7	15^{b} ±1	16^{b} ±.6	19^{b} ±2	18^{b} ±1	17^{b} ±1
2	98^{c} ±8	128^{c} ±11	110^{c} ±8	130^{c} ±13	171^{c} ±11	179^{c} ±13	205^{c} ±16	205^{c} ±17	187^{c} ±14	180^{c} ±13	205^{c} ±16
3	143^{c} ±14	142^{c} ±13	133^{c} ±13	130^{c} ±11	158^{c} ±13	167^{c} ±12	188^{c} ±14	168^{c} ±13	170^{c} ±13	164^{c} ±12	175^{c} ±14
Significance Level											
TRT	-	-	-	.08	.03	.02	.02	.03	.03	.03	.03

[a]Values represent means and standard errors of plasma cholesterol concentrations of rabbits corresponding to days of experiment.

[b-c]Means in the same column with the same superscript are not significantly different ($P < 0.05$).

Bacterial feeding was initiated on day 28 and terminated on day 49. Feeding of the 0.1% cholesterol-enriched diet continued until day 77. Groups: 1 = No cholesterol (control), N = 4; 2 = 0.1% cholesterol + boiled *E. coprostanoligenes* in 2% fat milk, N = 6; 3 = 0.1% cholesterol + *E. coprostanoligenes* in 2% fat milk, N = 8.

Table 4. Changes in plasma cholesterol concentrations from day 28 of rabbits fed a commercial chow followed by a 0.1% cholesterol-enriched diet and *Eubacterium coprostanoligenes* in 2% fat milk for 21 days

Changes in Plasma Cholesterol Concentrations[a] (mg/100 ml)							
	Days of Experiment						
Groups	35	42	49	56	63	70	77
1	1.0[b]	0.5[b]	-1.7[b]	1.6[b]	2.7[b]	-0.6[b]	-1.4[b]
2	40.4[c]	7.5[b]	26.9[b]	-1.1[b]	-17.8[b]	-7.4[b]	25.3[b]
3	28.8[bc]	7.7[b]	21.8[b]	-20.1[b]	1.7[b]	-5.9[b]	11.5[b]
Significance Level							
TRT	.13	.78	.23	.26	.89	.03	.19

[a]Values represent mean changes from day 28 in plasma cholesterol concentrations of rabbits corresponding to days of experiment.
[b-c]Means in the same column with the same superscript are not significantly different ($P < 0.05$)).
Bacterial feeding was initiated on day 28 and terminated on day 49. Feeding of the 0.1% cholesterol-enriched diet continued until day 77. Groups: 1 = No cholesterol (control), N = 4; 2 = 0.1% cholesterol + boiled *E. coprostanoligenes* in 2% fat milk, N = 6; 3 = 0.1% cholesterol + *E. coprostanoligenes* in 2% fat milk, N = 8.

Total plasma cholesterol concentration in rabbits in group 3 fed the 0.1% cholesterol + *E. coprostanoligenes* on days 0-77 and days 28-77 were not significantly different ($P < 0.05$) from that of group 2 fed the 0.1% cholesterol + boiled *E. coprostanoligenes* (Table 5). Changes in plasma cholesterol concentrations for days 0-77 were statistically significant ($P< 0.01$) and changes in plasma cholesterol for days 28-77 were different at $P< 0.14$ (Table 6)(Figure 2).

Differences in plasma cholesterol concentrations were observed in rabbits in groups 2 and 3 after feeding the regular commercial chow followed by the 0.1% cholesterol-enriched diet. Plasma cholesterol concentrations decreased in all groups when fed the commercial chow (days 77 to 108). Total plasma cholesterol concentrations in treated rabbits increased when the 0.1% cholesterol-enriched diet was fed (days 108 to 137) (Figure 1). Total plasma cholesterol concentration in rabbits in group 3 fed the 0.1% cholesterol + *E. coprostanoligenes* remained lower than those in the group 2 fed the 0.1% cholesterol + boiled *E. coprostanoligenes*, but the differences were not statistically significant ($P< 0.05$) (Table 6).

Results of analysis of plasma biochemical parameters evaluated are shown in Table 7. No significant changes ($P< 0.05$) were observed in plasma GOT, GPT, LDH and ALP activities and bilirubin and uric acid concentrations during the bacterial feeding period in rabbits group 3 fed the live or

Table 5. Plasma cholesterol concentrations of rabbits fed a commercial chow followed by a 0.1% cholesterol-enriched diet and *Eubacterium coprostanoligenes* in 2% fat milk for 21 days

Total Plasma Cholesterol Concentrations[a] (mg/100 ml)						
	Periods of Experiment (days)					
Groups	0-28	35-49	56-77	28-77	0-77	108-137
1	17.9[b]	15.8[b]	17.7[b]	16.8[b]	17.4[b]	20.3[b]
2	135.3[c]	185.3[c]	194.0[c]	190.2[c]	163.4[c]	140.3[c]
3	122.6[c]	170.9[c]	169.0[c]	169.9[c]	158.0[c]	115.2[c]
Significance Level						
TRT	.083	.022	.028	.14	.035	.144
DAY	.235	.0016	.102	.002	.0001	.001
TRT*DAY	.487	.279	.39	.38	.0003	.749

[a]Values represent means of plasma cholesterol concentrations of rabbits corresponding to days of experiment.
[b-c]Means in the same column with the same superscript are not significantly different ($P < 0.05$).
Bacterial feeding was initiated on day 28 and terminated on day 49. Feeding of the 0.1% cholesterol-enriched continued until day 77. Groups: 1 = No cholesterol (control), N = 4; 2 = 0.1% cholesterol + boiled *E. coprostanoligenes* in 2% fat milk, N = 6; 3 = 0.1% cholesterol + *E. coprostanoligenes* in 2% fat milk, N = 8.

Table 6. Changes in plasma cholesterol concentrations during different periods of rabbits fed a commercial chow followed by a 0.1% cholesterol-enriched diet and *Eubacterium coprostanoligenes* in 2% fat milk for 21 days

Changes in Plasma Cholesterol Concentrations[a] (mg/100 ml)						
	Periods of Experiment (Days)					
Groups	0-28	35-49	56-77	28-77	0-77	108-137
1	-1.6[b]	0.1[b]	0.6[b]	0.3[b]	-.28[b]	9.6[b]
2	11.0[b]	24.9[b]	-.2[b]	10.6[c]	10.69[c]	5.2[b]
3	4.2[b]	19.3[b]	-3.2[b]	6.9[bc]	3.27[b]	1.6[b]
Significance Level						
TRT	.09	.15	.325	.14	.011	.560
DAY	.04	.009	-.07	.0001	.0001	.003
TRT*DAY	.22	.43	-.31	.204	.172	.223

[a]Values represent mean changes of plasma cholesterol concentrations of rabbits corresponding to periods of experiment.
[b-c]Means in the same column with the same superscript are not significantly different ($P < 0.05$).
Bacterial feeding was initiated on day 28 and terminated on day 49. Feeding of the 0.1% cholesterol-enriched diet continued until day 77. Groups: 1 = No cholesterol (control), N = 4; 2 = 0.1% cholesterol + boiled *E. coprostanoligenes* in 2% fat milk, N = 6; 3 = 0.1% cholesterol + *E. coprostanoligenes* in 2% fat milk, N = 8.

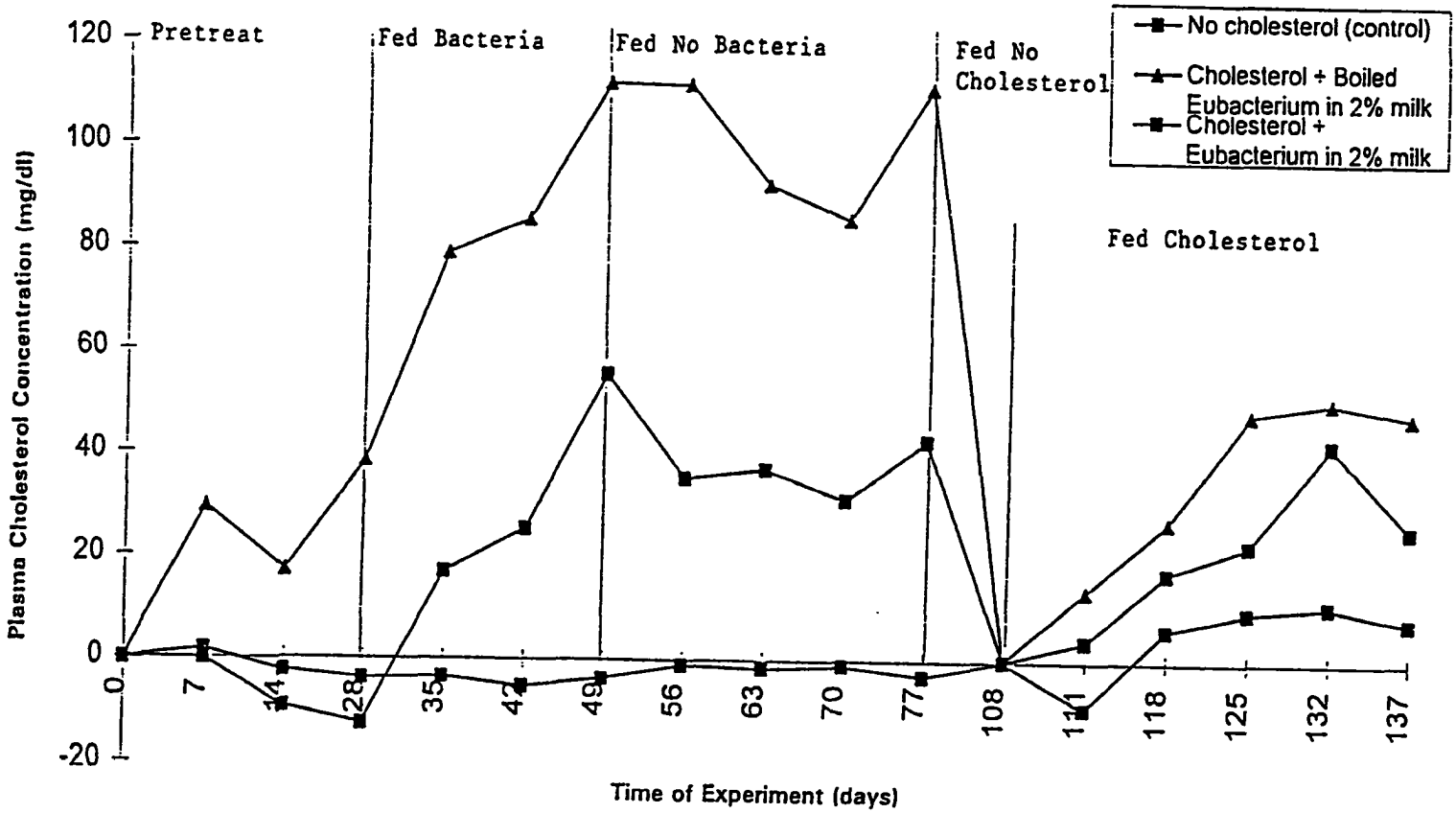

Figure 2. Total changes in plasma cholesterol concentrations observed in hypercholesterolemic rabbits after daily orally administrated *Eubacterium coprostanoligenes*. All rabbits except control received a 0.1% cholesterol-enriched before being assigned to the experimental groups at day 0. 5 ml of a 2% fat milk was used to deliver the bacteria to the rabbits. Each data point represents the group average change of triplicate assays of plasma samples from each rabbit. *Eubacterium* was fed from day 28-49. Feeding of the 0.1% cholesterol-enriched diet was continued through day 77). Regular chow was fed from day 78 to day 108, then followed by 0.1% cholesterol-enriched diet from day 109 to day 137.

group 2 fed the boiled bacteria when compared with those parameters of control rabbits in group 1. GOT activities showed small differences between treated rabbits and controls on day 42 ($P < 0.10$), but no significant differences were observed at day 56 ($P < 0.05$).

Total plasma protein concentrations remained constant throughout the experiment (Table 7). Significant differences ($P < 0.002$) were observed in bilirubin concentrations in rabbits in group 2 fed the fed 0.1% cholesterol-enriched diet + boiled *Eubacterium coprostanoligenes* when compared with those rabbits in group 3 fed the live bacteria and control rabbits in group 1 at the initiation of bacterial feeding.

Fourteen days after initiation (day 42) of bacterial feeding, bilirubin concentration in rabbits in group 3 fed the live bacteria was significantly higher ($P < 0.001$) than that in rabbits in group 2 fed the boiled bacteria and in the control rabbits, but the value was the same as that recorded for group 3 at the beginning of the experiment (Table 7).

No significant differences ($P < 0.05$) were observed in bilirubin concentration between treatment groups one week after termination of bacterial feeding. Uric acid concentration was significantly higher ($P < 0.04$) in rabbits group 3 fed the live bacteria when compared with that of rabbits in group 3 fed the boiled bacteria and with that of rabbits in the control group, but the value was lower than that recorded for group 3 at the initiation of bacterial

Table 7. Plasma enzyme activities and metabolite concentrations[a] of rabbits fed a commercial chow followed by a 0.1% cholesterol-enriched diet and *Eubacterium coprostanoligenes* in 2% fat milk for 21 days

Groups	GOT	GPT	LDH	ALP	Total Protein	Bilirubin	Uric acid
	(IU/L)				(g/100 ml)	(mg/100 ml)	
Day 28 - initiation of bacterial feeding							
1	12±1[b]	27±4[b]	236±34[b]	0	5±.3[b]	0.10±.03[b]	0.55±.03[b]
2	14±.8[b]	30±1[b]	205± 6[b]	3±.3[b]	6±.3[b]	3.5±1[c]	0.45±.3[b]
3	14±.9[b]	21±.1[b]	215± 7[b]	8±.9[b]	6±.1[b]	0.20±.1[b]	2.50±.4[b]
Day 42 - 14 after initiation of bacterial feeding							
1	9±1[b]	30±4[b]	123±37[b]	0	5±.1[b]	0.10±0[b]	0.15±.1[b]
2	17±1[c]	29±.5[b]	246± 9[b]	3±.7[b]	6±.1[b]	0.10±.1[b]	0.55±.1[bc]
3	13±.4[bc]	23±.5[b]	238±16[b]	3±3[b]	6±.1[b]	0.20±.01[b]	0.78±.04[c]
Day 56 - 7 days after cessation of bacterial feeding							
1	13±2[b]	29±7[b]	294±4[b]	0.3±.1[b]	5±.1[b]	0.10±.03[b]	0.10±.03[b]
2	20±2[b]	25±2[b]	244±23[b]	3.0±.2[b]	6±.1[b]	0.20±.02[b]	0.40±.1[b]
3	11±2[b]	14±2[b]	346±5[b]	0.8±.3[b]	6±.1[b]	0.60±.10[b]	0.20±.1[b]

[a]Values represent means and standard errors of plasma enzyme activities and metabolite concentrations per group of rabbits corresponding to days of experiment

[b-c]Means in the same column within the same period with common superscript are not significantly different ($P < 0.05$)

Groups: 1 = No cholesterol (control), N = 4; 2 = 0.1% cholesterol + boiled *E. coprostanoligenes* in 2% fat milk, N = 6; 3 = 0.1% cholesterol + *E. coprostanoligenes* in 2% fat milk, N = 8.

GOT = glutamic oxaloacetic transaminase; GPT = glutamic pyruvic transaminase; LDH = lactic dehydrogenase; ALP = alkaline phosphatase.

feeding. No significant differences ($P< 0.05$) were observed between treatment groups one week after termination of bacterial feeding (Table 7). Overall, the results suggest that the bacteria did not cause any detectable changes in liver function or damage.

Liver damage usually results in elevated plasma GOT, GPT, LDH and ALP activities and decreased plasma total protein concentrations. Hepatic excretory dysfunction usually shows increased ALP activities and small elevations in bilirubin concentrations (28,29). Increased GOT activities and uric acid concentrations and hypoproteinemia are suggestive of nephrotoxic manifestations. GOT activities and uric acid concentration at day 42 were not different from those at day 28 and were not accompanied by changes in total protein which remained constant through the experiment.

Susceptibility of the rabbits to the effects of the bacteria should depend not only on the nutritional status of the rabbits but also on the amount of bacteria incorporated into the diet, the ability of the bacteria to colonized the intestinal tract and to produce metabolites that are toxic to the rabbits and the rate of detoxification of any toxic metabolites.

The results of the evaluation of biochemical and hematological parameters further suggest that these bacteria are non-toxic and non-pathogenic. It may be possible to incorporate *E. coprostanoligenes* into foods commonly consumed

by humans without changing their nutritional, aesthetic and palatable qualities. This possibility could result in development of technology to use cholesterol reductase produced by *E. coprostanoligenes* to decrease the cholesterol content of foods and the possibility for treatment of hypercholesterolemia in humans.

The major sterols detected in feces of rabbits fed live and dead *E. coprostanoligenes* were cholesterol and coprostanol (Table 8) and ß-sitosterol, whereas the major bile acid was deoxycholic acid (Table 9). Rabbits in the group 2 fed the live bacteria excreted the highest fecal cholesterol concentration at 21 days before bacterial feeding when compared with that of rabbits in the other groups, but no coprostanol was detected in feces of rabbits from the other two groups. There were no significant differences ($P < 0.05$) in cholesterol concentration in excreta of rabbits at the start of the bacterial feeding (Table 8).

Fecal cholesterol concentrations increased at two weeks of bacterial feeding in rabbits fed both the live and dead bacteria, whereas cholesterol concentration decreased in the control rabbits. Cholesterol concentrations in excreta of rabbits in group 2 fed the dead bacteria increased 1.95 times compared with 1.67 times in rabbits in group 3 fed the live bacteria at 2 weeks (day 42) of bacterial feeding when compared with concentrations at the initiation of bacterial feeding. The differences in cholesterol concentration were not

Table 8. Fecal cholesterol and coprostanol concentrations[a] and weight ratios of rabbits

Groups	N	Cholesterol(Chol)	N	Coprostanol(Cop)	Cop/Chol
		(ug/g)			
		Day 0- 21 days before bacterial feeding			
1	3	15.1 ± 4.5 [b]	4	---	----
2	5	27.3 ± 0.4 [b]	6	---	----
3	8	33.6 ± 2.5 [b]	2	6.5 ± 1.7	0.19
		Day 28 - Initiation of bacterial feeding			
1	4	138.2 ± 8[b]	4	127.1 ± 17.2[b]	0.92
2	6	246.4 ± 19.8[b]	6	160.9 ± 11.1[b]	0.65
3	8	232.9 ± 11.7[b]	8	119.6 ± 9.6[b]	0.33
		Day 42- 14 days of bacterial feeding			
1	4	104.3 ± 7.7[b]	2	59.4 ± 8.4[b]	0.57
2	6	480.6 ± 28.7[c]	6	127.2 ± 12.6[b]	0.26
3	8	388.2 ± 20.9[c]	5	61.7 ± 5.7[b]	0.16
		Day 56- 7 days after cessation of bacterial feeding			
1	4	117.2 ± 6.7[b]	4	128.5 ± 17.0[b]	1.10
2	6	167.9 ± 16.7[b]	6	122.7 ± 7.9[b]	0.73
3	8	139.0 ± 8.7[b]	8	58.8 ± 3.1[c]	0.42
		Day 77- 28 days after cessation of bacterial feeding			
1	4	58.0 ± 13.7[b]	3	24.7 ± 3.2[b]	0.42
2	6	82.1 ± 4.9[b]	5	35.6 ± 4.5[b]	0.43
3	8	172.5 ± 17.7[b]	8	123.7 ± 20.6[b]	0.72
		Day 132- 24 days of refeeding of 0.1% cholesterol diet			
1	3	61.9 ± 31.1[b]	2	35.6 ± 15[b]	0.57
2	6	225.4 ± 9.8[c]	6	147.8 ± 20.9[b]	0.66
3	8	191.2 ± 11.8[c]	8	60.5 ± 4[b]	0.32
		Day 137- 29 days of refeeding of 0.1% cholesterol diet			
1	2	76.4 ± 9.6[b]	2	62.9 ± 22.4[b]	0.82
2	6	156.2 ± 22.5[b]	6	140.3 ± 26.6[b]	0.92
3	8	116.3 ± 7.2[b]	6	58.5 ± 6.2[b]	0.50

[a]Values represent means and standard errors of fecal cholesterol and coprostanol concentrations from rabbits.
[b-c]Means in the same column within the same time period with same superscript are not significantly different ($P < 0.05$).
Groups: 1 = No cholesterol (control); 2 = 0.1% cholesterol + boiled *E. coprostanoligenes* in 2% fat milk; 3 = 0.1% cholesterol + *E. coprostanoligenes* in 2% fat milk
--- = No detectable amount

Table 9. Fecal ß-sitosterol and deoxycholic acid concentrations[a] in feces of rabbits

Groups	N	ß-Sitosterol (ug/g)	N	Deoxycholic acid (ug/g)
		Day 0- 21 days before bacterial feeding		
1	3	33.6 ± 18.5[b]	4	39.0 ± 3.0[b]
2	4	99.9 ± 38.0[b]	6	31.1 ± 2.7[b]
3	6	45.8 ± 8.7[b]	8	47.5 ± 3.6[b]
		Day 28 - Initiation of bacterial feeding		
1	4	1073.2 ± 311.8[b]	4	223.5 ± 61.6[b]
2	6	1369.7 ± 211.2[b]	6	222.8 ± 21.3[b]
3	8	1495.1 ± 107.4[b]	8	260.1 ± 17.2[b]
		Day 42- 14 days of bacterial feeding		
1	4	1688.7 ± 129.7[b]	4	233.6 ± 10.1[b]
2	6	2280.9 ± 129.4[b]	6	258.4 ± 12.3[b]
3	8	1765.3 ± 81.4[b]	8	268.1 ± 12.6[b]
		Day 56- 7 days after cessation of bacterial feeding		
1	4	2044.1 ± 55.7[b]	4	278.8 ± 6.6[b]
2	5	1759.9 ± 123.7[b]	5	214.9 ± 14.0[bc]
3	8	1381.2 ± 84.0[b]	8	171.6 ± 9.2[b]
		Day 77- 28 days after cessation of bacterial feeding		
1	4	1776.4 ± 283.1[b]	4	243.1 ± 26.3[b]
2	6	1559.2 ± 76.0[b]	6	223.0 ± 12.6[b]
3	8	2829.1 ± 183.0[b]	8	413.4 ± 23.7[c]
		Day 132- 24 days of refeeding of 0.1% cholesterol diet		
1	3	265.3 ± 127.5[b]	4	198.7 ± 59.6[b]
2	4	692.9 ± 27.1[bc]	6	393.8 ± 11.9[b]
3	6	818.1 ± 62.5[c]	8	479.5 ± 31.7[b]
		Day 137- 29 days of refeeding of 0.1% cholesterol diet		
1	4	1702.4 ± 60.0[b]	4	384.1 ± 46.5[b]
2	6	1120.1 ± 698.9[b]	6	315.3 ± 31.1[b]
3	8	1055.7 ± 81.2[b]	8	405.5 ± 24.7[b]

[a]Values represent means and standard errors of fecal bile acid concentrations from rabbits.
[b-c]Means in the same column within the same time period with same superscript are not significantly different ($P < 0.05$).
Groups: 1 = No cholesterol (control); 2 = 0.1% cholesterol + boiled *E. coprostanoligenes* in 2% fat milk; 3 = 0.1% cholesterol + *E. coprostanoligenes* in 2% fat milk.

statistically significant (P< 0.05)(Table 8).

On day 77 (4 weeks after bacterial feeding), rabbits in group 3 fed the live bacteria excreted feces with greater concentrations of cholesterol than rabbits fed boiled bacteria (P< 0.14). No significant differences (P< 0.05) were observed in cholesterol excreted in feces of rabbits fed the live bacteria and rabbits fed the boiled bacteria on days 132 and 137 (Table 8). Investigation of oral administration of *Streptococcus feacalis KAWI* powder to rats fed a 0.5% cholesterol diet increased cholesterol excreted in feces by 65% and 39% after 2 and 4 weeks, respectively (15).

Coprostanol concentration was significantly lower (P< 0.02) at day 56 in rabbits in group 3 fed the live *E. coprostanoligenes* bacteria when compared with rabbits in group 2 fed the boiled *E. coprostanoligenes* bacteria (Table 8). Rabbits fed the live bacteria had 2.1 times higher cholesterol and 4.1 times higher coprostanol concentrations excreted in feces at 4 weeks after bacterial feeding (day 77) when compared with rabbits fed the boiled bacteria (Table 8).

During the bacterial feeding period coprostanol, concentrations and coprostanol/cholesterol concentration ratios decreased in all groups of rabbits. The coprostanol/ cholesterol concentration ratio in rabbits fed the live bacteria at day 77 was 1.7 times greater in rabbits fed the live bacteria when compared with rabbits fed the boiled bacteria (Table 8).

The excretion of sitosterol and bile acids increased in all groups of rabbits at 2 weeks of bacterial feeding and 4 weeks after bacterial feeding when compared with the fecal concentrations at the initiation of bacterial feeding (Table 8). Rabbits fed the dead bacteria excreted 1.29 times more sitosterol and 0.98 times more bile acids than rabbits fed the dead bacteria at 2 weeks after bacterial feeding. Bile acid excretion was significantly lower ($P< 0.05$) in rabbits fed the live bacteria on day 56 when compared with rabbits fed the boiled bacteria (Table 9). Rabbits fed the live bacteria excreted significantly higher ($P< 0.05$) concentrations of bile acid than rabbits fed the boiled bacteria at day 77. At day 132 and 137 rabbits fed the live bacteria excreted higher bile acid than rabbits fed the boiled bacteria, but differences were not statistically significant ($P< 0.05$) (Table 9).

Studies of sterol and bile acid excretion in hypercholesterolemia-resistant rabbits showed that resistant rabbits when compared to normoresponsive rabbits excreted significantly larger amounts of fecal bile acids. Preliminary tissue distribution studies 24 hours after consumption of cholesterol-supplemented diet containing [4-^{14}C]cholesterol bile aspirated from gallbladder at necropsy contained an average of 2.2 times more radioactivity than specimens obtained from normoresponsive rabbits (22).

Rabbits fed the live bacteria excreted 1.81 times more sitosterol and 1.85 times more bile acid than rabbits fed the

boiled bacteria at 4 weeks after termination of bacterial feeding. Sitosterol excreted by rabbits fed the live bacteria was greater at day 77 when compared with that excreted by rabbits fed the boiled bacteria, but the differences were not significantly different ($P < 0.05$)(Table 9).

CONCLUSIONS

Oral administration of *E. coprostanoligenes*, a cholesterol-reducing bacteria, in 2% fat milk decreased cholesterol concentrations in New Zealand White adult male rabbits receiving a 0.1% cholesterol-enriched diet. No clinical signs or symptoms of bacterial intoxication were observed in any rabbit. Feeding of the bacteria did not affect rabbit mean body weight. Changes in plasma cholesterol concentration from days 0-77 in rabbits fed the 0.1% cholesterol + live *E. coprostanoligenes* in 2% milk diet were lower ($P < 0.01$) than plasma cholesterol concentration in rabbits fed the 0.1% cholesterol + boiled *E. coprostanoligenes* in 2% milk diet.

Incorporation of *E. coprostanoligenes* in milk and feeding to New Zealand White adult male rabbits receiving a 0.1% cholesterol-enriched has been shown to be non-toxic and non-pathogenic to rabbits because no changes were observed in plasma enzymes (GOT, GPT, LDH and ALP) activities and major metabolites (bilirubin, uric acid and total protein) concentrations during the bacterial feeding when compared with

corresponding activities and concentrations at the initiation of the bacterial feeding.

The results suggest that *E. coprostanoligenes* is non-toxic and non-pathogenic in rabbits and may be safe for human consumption. Results further suggest that milk may be used as a medium to deliver a cholesterol-reducing microbe to decrease plasma cholesterol concentration in humans. Therefore, the decrease in plasma cholesterol concentrations observed in hypercholesterolemic rabbits fed *E. coprostanoligenes* may have important positive impacts on human health and welfare.

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ADDITION OF *EUBACTERIUM COPROSTANOLIGENES* TO DECREASE THE CHOLESTEROL CONTENT OF FERMENTED PORK AND MUTTON SAUSAGES

A paper to be submitted to the Journal of Food Science

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Abstract

Three preliminary investigations of the potential use of *Eubacterium coprostanoligenes*, a cholesterol-reducing bacteria, with or without commercially available *Lactobacillus* starter culture in the production of fermented pork and mutton sausages were conducted. Meat samples were assigned to the following treatment groups; uninoculated (control), *Lactobacillus* commercial starter culture, various combinations of *Lactobacillus* + *E. coprostanoligenes* and *E. coprostanoligenes*. All treatments were allowed to undergo the normal fermentation process at 37° C for 12-15 hours, then the sausages were cooked at 68° C in conventional oven. The viability of *E. coprostanoligenes* after fermentation was assessed by culturing samples of meat from each treatment. All sausages were evaluated for cholesterol and coprostanol concentrations, moisture, total lipid and pH before and after fermentation. After fermentation pH decreased in sausages inoculated with *Lactobacillus* and the various combinations of

Lactobacillus + *E. coprostanoligenes*, were significantly lower ($P < 0.05$) than that of control and the other treatments. Moisture, total lipid and cholesterol content in fermented sausages were not consistently affected by the bacterial treatments.

Coprostanol was detected only in cultures from fermented sausages inoculated with the 0.5 g of *E. coprostanoligenes* pellet in experiment 2. In experiment 3, coprostanol was detected in all treatments after fermentation except uninoculated (control) pork sausages. Significantly greater ($P < 0.05$) concentrations of coprostanol were detected in fermented sausages and cultures from fermented sausages inoculated with the 2.0 g of *E. coprostanoligenes*. Detection of coprostanol in fermented sausages and in cultures from fermented sausages confirmed that *E. coprostanoligenes* remained viable during fermentation while converting cholesterol to coprostanol and maintained its ability to reduce cholesterol to coprostanol when added to media containing 0.2% cholesterol.

INTRODUCTION

Consumers worldwide have become more concerned the composition of their diets and how changes in life styles can result in lower blood cholesterol concentration and thus lower

risk of coronary heart disease. Studies have demonstrated that there is a causal association between serum cholesterol concentration, dietary fat, and coronary heart disease, and it is hypothesized that the risk of heart disease should be reduced substantially when serum cholesterol is lowered (Ravnskov, 1992). Cholesterol content of animal-derived foods is a primary public concern as there is considerable evidence to suggest that plasma cholesterol plays an important role in the development of atherosclerosis and coronary heart disease.

Cholesterol is required for several biological functions in animals, including humans; precursor for biosynthesis of bile acids, corticosteroids and sex hormones; regulation of membrane activity and transport of lipids from the intestines to other tissues; and proliferation of a variety of cells (Dehal et al., 1991). The greatest challenge to meat scientist is to lower the palmitic acid content, the major fatty acid in meat and meat products, which is associated with increase in plasma cholesterol concentration in humans (Grundy, 1986). Consumers prefer foods with low lipid content and producers have need for application of inexpensive methods to reduce excess fat and cholesterol in various meat and meat-products without changing the nutritional, aesthetic and palatability qualities.

In studies conducted with starter cultures on quality of fermented mutton sausages, starter cultures did not affect

water activity, ash or protein content, fatty acid composition, cholesterol or retention of moisture, protein fat, ash or fatty acids (Long et al., 1990). Production of Manchego cheese with fast acid producing bacteria may cause bitterness during ripening (Nunez et al., 1982). Starter cultures prepared with nonbitter strains may be used to minimize bitterness in cheese when acid production is not necessary for cheese production (Wu et al., 1991). Starter cultures that produce antimicrobial compounds may be beneficial for both consumer safety and shelf life of product (Nunez et al., 1982).

Numerous chemical, physical and biological methods have been employed to decrease or eliminate fat and cholesterol in milk because of the perceived association of dairy products with cardiovascular disease (Chavarri et al., 1988). Physical and chemical methods tend to be nonselective and usually remove the nutritional components with cholesterol (Deeth, 1983). The use of organic solvents results in residues in the final product. Supercritical fluid extraction, vacuum steam distillation, short path distillation, and complexing with cyclodextrins and saponins have been used also (Deeth, 1983; Smith et al., 1991). Bacterial and enzyme methods offer potential cost and selectivity advantages (Deeth, 1983). Degradation and reactivity of cholesterol in milk have been studied at different temperatures using bacterial cholesterol

oxidases (Deeth, 1983; Smith et al., 1991).

Demonstrations have shown that bacteria as well as plants can metabolize steroids by transformation of their side chains and steroid nuclei to produce pharmaceutically active steroid compounds (Dehal et al., 1991). *Lactobacillus acidophilus* RP32 significantly inhibited increase in serum cholesterol concentrations in pigs fed high-cholesterol diet (Xiansheng et al., 1990). Hydrogenation of cholesterol by anaerobic incubation with rumen fluid for 20 hours resulted in coprostanol as the principal product (Eyssen et al., 1973). Results of catalytic hydrogenation of cholesterol indicated that coprostanol was one of the by-products (Ashes et al., 1978).

Escherichia coli in human feces was found to degrade cholesterol under aerobic and anaerobic conditions to form neutral products, cholest-4 en-3-one, cholesta-1,4-dien-3-one, androst-4-ene-3,17-dione and androsta-1,4-diene-3,17-dione. Cholesta-1,4-dien-3-one and androsta-1,4-diene-3,17-dione were also produced under anaerobic condition (Hershberg et al., 1951). Degradation of cholesterol by *Pseudomonas species NCIB 10590* showed that the major products included cholest-5-en-3-one, cholest-4-en-3-one, 26-hydroxycholest-4-en-3-one, androst-1,4-dien-3,17-dione, cholest-4-en-3-one-26-oic acid, chol-4-en-3-one-24-oic acid, pregn-4-en-3-one-20-carboxylic acid and pregna-1,4-dien-3-one-20 carboxylic acid (Owen et

al., 1978).

Microbial hydrogenation of cholesterol to coprostanol may occur by the intermediates 4-cholesten-3-one and coprostanone (Owen et al., 1983). Microbial transformation of cholesterol by cecal contents from rats showed that at least 50% of the cholesterol converted to coprostanol by means of the intermediate 4-cholestenone (Bjorkhem and Gustafsson, 1971).

In the human gut, cholesterol was partially degraded to coprostanol and coprostanone and determination of metabolites is complicated by the degradation of products from plant sterols (Macdonald et al., 1983). *Mycobacterium* species were magnetically immobilized and used for side chain degradation of cholesterol into androsta-1,4-dien-3,17-dione (Flygare and Larsson, 1987).

Eubacterium can anaerobically reduce cholesterol to coprostanol, which is absorbed poorly by the human gastrointestinal tract (Gilliland et al., 1984). *Eubacterium 21,408* reduced the 4,5 double band of allocholesterol to coprostanol (Gilliland et al., 1984).

Freier, (1991) successfully isolated a pure culture of *Eubacterium coprostanoligenes* and it was characterized as a small, gram positive, nonspore forming, coccobacillus, cholesterol-reducing anaerobe. Lecithin is required for growth, whereas cholesterol is not. Coprostanol is not produced when lecithin is replaced by phosphatidylinositol or

phosphatidylglycerol. *E. coprostanoligenes* possesses phospholipase activity and does not reduce nitrate or produce indole. Esculin was hydrolyzed, but starch and gelatin were not. *E. coprostanoligenes* produced much acid by fermentation of amygdalin, lactose and salicin (acetic, formic and succinic), whereas arabinose, cellobiose, fructose, glucose, mannose and melibiose were poorly fermented. Volumes of gases produced in the head space showed that H_2 and CO_2 percentages ranged from 4.5 to 7.2 and 0.9 and 1.8, respectively (Frier, 1991, Frier et al., 1994). Fermentation of radiolabelled [4-^{3}H, 4-^{14}C]-cholesterol with *E. coprostanoligenes* resulted in 90% conversion of cholesterol to coprostanol in 5 days. Coprostanol formation by *E. coprostanoligenes* involved the transfer of a hydrogen from C4 to C5 position. *E. coprostanoligenes* reduction of radioactive coprostanone to coprostanol tend to favor the theory of the indirect pathway with the isomerization of a double bond at the 4-5 position (Ren, 1991).

Oral administration of *E. coprostanoligenes* to New Zealand White rabbits fed a 0.05% cholesterol-enriched diet resulted in lower ($P < 0.001$) plasma cholesterol concentration in rabbits fed the live bacteria for 10 days when compared with rabbits fed the boiled bacteria. Coprostanol/cholesterol ratios in the contents of the digestive tracts of rabbits fed the live bacteria were greater than those of rabbits fed the

boiled bacteria (Li et al., 1995).

The hypothesis of this study was that *Eubacterium coprostanoligenes* could grow in meats during sausage production and would decrease the cholesterol content of meats. The studies were undertaken to investigate the feasibility of using a cholesterol-reducing bacteria in the production of pork and mutton sausages. We evaluated the viability of *E. coprostanoligenes* in the fermented pork and mutton sausages and the concentrations of cholesterol and coprostanol, total lipids, moisture and pH at different stages of production and storage of the sausages.

MATERIALS AND METHODS

Preparation of starter culture

Eubacterium coprostanoligenes ATCC 51222 was maintained in 10 ml cultures grown anaerobically in liquid media composed of 0.1% lecithin (Sigma Chemical Co. St. Louis, MO), 1% casitone, 1% yeast extract (Difco Laboratories, Detroit, MI), 0.5% sodium thioglycollate and 0.1% calcium chloride (Fisher Scientific, Fair Lawn, NJ). Resazurin added at 0.4% as an indicator for the presence of oxygen. Lacto-Bacto plates prepared from media containing 1% Bacto-Tryptone, 0.5% Bacto-Yeast, 1% sodium chloride and 1.5% Bacto-Agar (Difco Laboratories, Detroit, MI) were used to monitor purity of

cultures. A 4 ml aliquot of *Eubacterium coprostanoligenes* stock culture was used to inoculate 400 ml flasks of media similar to the maintenance as indicated above by using the Hungate technique (Holdeman et al., 1977). Each flask was sealed with a rubber stopper and incubated at 37° C for 48 hours for growth of the bacteria used to inoculate meat samples. The *Lactobacillus* commercial starter culture used was Lactacel^{R} 115 (Microlite Technics, Sarasota, FL).

Preparation of ground pork and mutton

Fresh ground pork was used in experiment one and two and ground pork and mutton were used in experiment three representing the normal consumer choice. The pork and mutton were ground through a 0.32 cm plate and mixed thoroughly to homogenize. Duplicate or triplicate samples were taken for analysis of pH, moisture, total lipids, cholesterol and coprostanol content before seasoning. The bulk meats were seasoned (3% sodium chloride, 0.75% dextrose, 0.015% sodium nitrate, 0.38% pepper), and vacuum-mixed. Duplicate or triplicate samples were taken from each meat for analysis of pH, moisture, total lipids, cholesterol and coprostanol content after seasoning (before fermentation), after fermentation, after cooking and after 1 month of storage after cooking.

Experiment one

Triplicate 100 g samples of each meat were weighed into autoclaved 400 ml or 600 ml beakers and randomly assigned to each of following treatments in Table 1. Triplicate 100 g ground pork samples were inoculated with 1 ml, 2 ml and 10 ml aliquots of liquid *E. coprostanoligenes* culture. The remaining bacteria were harvested by centrifuging the 48 hour bacterial culture in pre-weighed bottles in a Sorvall^{R} RC2-B refrigerated centrifuge at 750 rpm for 20 minutes and the weight of the bacterial pellet was calculated and treatment doses determined. Bacterial pellet of *E. coprostanoligenes* was weighed in triplicate amounts of 0.05 g and 0.1 g and duplicate 0.2 g. Each amount of bacterial pellet was suspended in 1 ml volume of sterile phosphate-buffered saline (0.01 M $Na_2HPO_4.7H_20$ + 0.15 M NaCl, pH 7.5)(PBS) and used to inoculate a 100 g sample of ground pork. Triplicate ground pork samples were inoculated also with 0.05 g of *Lactobacillus* pellet and the combination 0.05 g of *Lactobacillus* + 0.05 g of *E. coprostanoligenes* pellets; uninoculated pork samples were used as controls. All samples were placed in jars and were incubated at 37° C for 12 hours. Treatments 3 and 4 were incubated under anaerobic conditions to facilitate the growth of *E. coprostanoligenes* by using gas jars charged with BBL^{R} GasPak Plus^{TM} system (Becton Dickinson, MD).

After fermentation, duplicate 0.5 g samples from sausages

Table 1. Assignment of meat samples

Treatments	Total Replicates
1. Uninoculated (control)	
Pork	3
2. *Lactobacillus* (0.05 g)	
Pellet - Pork	3
3. *Lactobacillus* + *E. coprostanoligenes* (0.05 of each)	
Pellet - Pork	3
4. *E. coprostanoligenes*	
Pork	
liquid media, 1, 2, 10 ml	9
pellet 0.05, 0.10, 0.2 g	9

from each treatment were suspended in a 1 ml volume of sterile PBS and each was used to inoculate a 9 ml volume culture containing the components of the media used to maintain the bacteria with cholesterol added at 0.2% for the evaluation of the viability of *E. coprostanoligenes*. All cultures from fermented sausages were incubated at 37° C for 96 hours. A 1 ml aliquot of each culture was taken for analysis of pH and a 1 ml aliquot was taken for analysis of cholesterol and coprostanol. A 1 g sample was taken from each pork sausage and used to determine pH and a 1 g sample was taken from each pork sausage to determine moisture content. A 0.5 g sample was taken from each pork sausage for analysis of total lipids, cholesterol and coprostanol concentrations.

Experiment two

The design of the experiment was similar to that in experiment 1 (Table 1). Triplicate 100 g ground pork samples were inoculated with 1 ml, 2 ml and 10 ml aliquot of bacterial culture. The remaining bacteria were harvested by centrifuging the 48 hour bacterial culture in pre-weighed bottles in a Sorvall^{R} RC2-B refrigerated centrifuge at 7,500 rpm for 20 minutes and the weight of the bacterial pellet was calculated and treatment loads determined. Bacterial pellet was weighed in triplicate amounts of 0.05 g, 0.1 g and duplicate 0.5 g. Each amount of bacterial pellet was suspended in a 1 ml volume of sterile PBS and used to inoculate a 100 g sample of ground pork. Triplicate ground pork samples were inoculated also with 0.05 g of *Lactobacillus* pellet and the combination of 0.05 g *Lactobacillus* + 0.05 g of *E. coprostanoligenes* pellets; uninoculated pork samples were used as controls. All samples were placed in jars and were incubated at 37° C for 15 hours. Treatments 3 and 4 were incubated under anaerobic conditions to facilitate the growth of *E. coprostanoligenes* by using gas jars charged with BBL^{R} GasPak Plus^{TM} system (Becton Dickinson, MD).

After fermentation, a 0.5 g of each pork sausage was suspended in a 1 ml volume of sterile PBS and each was used to inoculate a 9 ml volume culture containing the components of the media used to maintain the bacteria with cholesterol added

at 0.2% for the evaluation of the viability of *E. coprostanoligenes*. All cultures from the fermented sausages were incubated at 37° C for 96 hours. A 1 ml aliquot of each culture was taken for analysis of pH and a 1 ml aliquot was taken for analysis of cholesterol and coprostanol. A 1 g sample was taken from each pork sausage and used to determine pH and a 1 g sample was taken to determine moisture content. A 0.5 g of each sausage was taken for analysis of total lipids, cholesterol and coprostanol concentrations.

All sausages were cooked at 68° C for 25 minutes then stored in freezer. A 1 g sample was taken from each pork sausage and used to determine pH and a 1 g sample was taken to determine moisture content. A 0.5 g of each sausage was taken for analysis of total lipid, cholesterol and coprostanol concentrations. All sausages were vacuumed-packaged and held at typical retail display case temperature (approx. 25° C to simulate the current demand for shelf stable fermented products) and sampled after 1 month of storage after cooking for performing the above mentioned analyses.

Experiment three

The design of this experiment was a modification of the first 2 experiments (Table 1) with both ground pork and mutton. Based on the results obtained in experiment 2, only bacterial pellets were used to inoculate the ground pork and

mutton samples. The bacteria were harvested by centrifuging in pre-weighed bottles in a refrigerated Sorvall^{R} RC2-B centrifuge at 750 rpm for 20 minutes and weight of the bacterial pellet was calculated and treatment loads determined. Bacterial pellet was weighed in triplicate amounts of 0.25 g and 0.5 g and duplicate amounts of 2 g bacterial pellet for inoculation of pork samples and triplicate 0.5 g and 1 g amounts for inoculation of mutton samples.

Each amount of bacterial pellet was suspended in a 1.5 ml volume of sterile PBS and used to inoculate a 100 g sample of ground pork or mutton. Triplicate ground pork samples were inoculated also with 0.05 g of *Lactobacillus* pellet and the combinations of 0.1 g, 0.25 g and 0.5 g of *E. coprostanoligenes* + 0.05 g *Lactobacillus* pellets; uninoculated pork and mutton sausages were used as control. All samples were placed in jars and were incubated at 37° C for 15 hours. Treatments 3 and 4 were incubated under anaerobic conditions to facilitate the growth of *E. coprostanoligenes* by using gas jars charged with BBL^{R} GasPak Plus^{TM} system (Becton Dickinson, MD).

After fermentation, a 0.5 g of each pork or mutton sausage was suspended in a 1 ml volume of sterile PBS and each was used to inoculate a 9 ml volume culture containing the components of the media used to maintain the bacteria with cholesterol added at 0.2% for the evaluation of the viability

of *E. coprostanoligenes*. All cultures from fermented sausages were incubated at 37° C for 96 hours. A 1 ml aliquot of each culture was taken for analysis of pH and a 1 ml aliquot was taken for analysis of cholesterol and metabolites. A 1 g sample was taken from each pork or mutton sausage and used to determine pH and a 1 g sample was taken to determine moisture content. A 0.5 g sample of each pork or mutton sausage was taken for analysis of total lipids, cholesterol and coprostanol concentrations. All sausages were cooked at 68° C for 25 minutes then stored in freezer. A 1 g sample was taken from each meat sample and used to determine pH and a 1 g sample was taken to determine moisture content. A 0.5 g of each pork or mutton sausage was taken for analysis of total lipids, cholesterol and coprostanol concentrations. All sausages were vacuumed-packaged and held at typical retail display case temperature and at room temperature (approx. 25° C to simulate the current demand for shelf stable fermented products) and sampled after 1 month of storage after cooking for analysis of pH, moisture, total lipids and cholesterol and coprostanol concentrations.

Sample preparation and analytical procedures

Duplicate or triplicate 1 g samples were taken from the fresh ground raw pork or mutton, ground pork and mutton after seasoning (before fermentation) and from each pork or mutton

sausage after fermentation for the determination of pH and moisture content and a 0.5 g of each meat sample was taken for analysis of total lipid content and cholesterol and coprostanol concentrations. Each 1 g of sample for pH determination was suspended in a 10 ml aliquot of deionized water, vortexed for 15 seconds and pH of each suspension was measured with a model 701A digital ionalyzer pH meter (Orion Research^{R}). A 1 g sample of each meat sample was placed in a pre-weighed crucible and placed in oven at 100° C over night. Each crucible was removed from the oven and placed in desiccator and weighed for moisture determination (AOAC, 1980).

Extraction of cholesterol and coprostanol in each 1 ml sample of each culture from fermented pork or mutton sausages was performed by the addition of 5 ml of chloroform:methanol (2:1, v/v) (AOAC, 1990) to each 20 ml screw-cap test tube. Each sample was vortexed for 60 seconds, then centrifuged at 2,000 rpm for 20 minutes. The bottom (chloroform) layer was transferred quantitatively to a 2 dram vial that had been treated with a solution of 10% dimethylchlorosilane in toluene (silanized) (Nawrocki, 1985), then concentrated to dryness in a heated block set at 65° C under nitrogen gas.

Analyses of total lipid, cholesterol, coprostanol concentrations in fresh ground raw before seasoning and ground pork or mutton after seasoning (before fermentation),

fermented, cooked fermented and fermented cooked pork and mutton sausages after 1 month of storage were performed by removal of triplicate or duplicate 0.5 g sample of each treatment and each placed in a 30 ml extraction tube. Extraction of total lipids in each meat sample was performed by the addition of 18.5 ml of methanol:water (2.5:1, v/v) to each 30 ml extraction tube (Bligh and Dyer, 1959). Each sample was vortexed for 10 seconds, then 6.5 ml of chloroform was added to each sample and each sample vortexed for 1 minute.

Each sample was shaken on a Burrell wrist-action shaker overnight, then 7.5 ml of chloroform and 7.5 ml of aqueous 0.37% potassium chloride (KCl) solution were added to each sample. Each extraction tube was inverted and centrifuged at 2,000 rpm for 25 minutes. The top aqueous layer was removed by aspirating and discarded. Ten ml of aqueous 0.37% KCl solution was added to each extraction tube and each tube inverted then centrifuged at 2,000 rpm for 25 minutes. The top aqueous layer was removed by aspirating and the procedure repeated with the 10 ml of aqueous 0.37% KCl solution. The top aqueous layer of each sample was removed by aspirating and each sample was filtered in a pre-weighed scintillation vial by using a 4.5 cm glass micro-fiber filter paper (Whatman^{R}), a Buchner funnel and a suction flask. Each meat residue was washed with two four ml volumes of chloroform into the scintillation vial and each chloroform extract was concentrated to dryness in a

Brinkman^{R} SC/48R sample concentrator at 50° C under nitrogen gas. The scintillation vials were allowed to cool at room temperature. Each vial containing each lipid extract was weighed and lipid weight of each sample calculated and expressed as percentages on wet and dry matter basis.

Each lipid extract was dissolved in 1 ml of chloroform, vortexed and transferred to a 20 ml extraction tube. Extraction of cholesterol and coprostanol was performed by the addition of 2 ml of methanol:benzene (4:1, v/v) and 200 µl of acetyl chloride to each sample tube and capped tightly. All tubes were placed on a heatblock set at 100 ± 3° C for 1 hour, then the tubes were removed from the heatblock and allowed to cool to room temperature. Five ml of 6% K_2CO_3 solution and 2 ml of benzene were added to each sample and each vortexed at high speed for 1 minute. All samples were centrifuged in a IEC model centrifuge (International Equipment Company, Neeham, Mass) at 2,000 rpm for 20 minutes. The top (benzene) layer of each sample extract was transferred quantitatively with individual pasteur pipet to individual silanized vial (Nawrocki, 1985). All samples were concentrated to dryness under nitrogen gas in a pre-heated block set at 65° C.

In experiment 1 and 2, each meat or culture sample extract was dissolved in 200 µl of chloroform . Forty µl of each meat sample extract or 100 µl of each culture sample extract was transferred to a new silanized vial (Nawrocki, 1985) and 20 µg

of cholestane standard was added to one replicate of each sample as internal standard. Mixtures of 0.125 µg, 0.25 µg, 0.5 µg and 1.0 µg of cholestane, cholesterol, coprostanol and cholest-4 en-3-one standards were used for gas chromatography (GC) analysis of the various sterols. Each sample extract and each standard mixture was concentrated to dryness under nitrogen gas, then derivatized with 100 µl of silylation grade acetonitrile and 100 µl of 1,3 bis(trimethylsilyl) trifluoroacetamide with 1% trimethyl chlorosilane (Pierce, Rockford, IL). Each derivatized sample was quantitatively transferred to 200 µl inserts for GC analysis as trimethylsilyl ethers (Overturf et al., 1990; Martin et al., 1972; Subbiah et al., 1972).

Analyses of cholesterol and coprostanol concentrations in were done on a Hewlett-Packard 5830A gas chromatograph equipped with a flame ionization detector. Column temperature was set at 280° C, injector at 290° C, detector 290° C and maximum oven temperature at 300° C. Separation of cholesterol and coprostanol occurred on a 0.9 M, 0.64 mm ID glass column packed with 3% SP-2250 on 100/200 Supelcoport (Supelco^{R}, Bellefonte, PA), carrier gas was nitrogen at 60 ml/min, air at 240 ml/min and hydrogen at 60 ml/min. The detector was equipped with an integrator set at slope sensitivity of 1.5 and paper speed of 0.7 cm/min. Quantitation of trimethylsilyl ether derivatives was by standard curve and the concentration

of each sterol in each extract was calculated by using cholestane concentrations as internal standard.

In experiment 3, 40 ml of each meat sample or culture sample extract was transferred to silanized vial and each was concentrated to dryness under nitrogen gas in a pre-heated heatblock set at 65° C. Two µg of cholestane standard was added to one replicate of each sample. Mixtures of 0.01 µg, 0.02 µg, 0.04 µg, 0.08 µg and 0.16 µg of cholestane, cholesterol, coprostanol and cholest-4 en-3-one standards were used for GC analysis of the various sterols. Each sample extract or standard mixture was derivatized with 100 µl of silylation grade acetonitrile and 100 µl of 1,3 bis(trimethylsilyl) trifluoroacetamide with 1% trimethyl chlorosilane (Pierce, Rockford, IL).

Each derivatized sample extract was concentrated to dryness under nitrogen gas. Each sample extract was then redissolved in 20 µl of hexane, vortexed and 1.5 µl of each extract was used for GC analysis as trimethylsilyl ethers (Overturf et al., 1990; Martin et al., 1972; Subbiah et al., 1972). Analyses of cholesterol and coprostanol concentrations were done on a Tracor 540 gas chromatograph equipped with a flame ionization detector and split injection system, column temperature was set at 290° C, injector at 290° C, detector at 300° C. Separation of cholesterol and coprostanol occurred on a DB™-5, (30 M x 0.25 µm ID x 0.25 µm film thickness) nonpolar

bonded phase (5% phenyl)-diphenyl-dimethylpolysolaxne capillary column (J & W Scientific, Folsom, CA), carrier gas was helium with flow rate at 60 ml/min, air at 400 ml/min and hydrogen at 30 ml/min. The detector was equipped with a Shimadzu Chromatopac^{R} C-R3A integrator with slope sensitivity set at 0 and paper speed of 0.7 cm/min. Quantitation of trimethylsilyl ether derivatives was done by comparing the area of each meatabolite in each sample extract to the area of a known concentration of each sterol standard in each control extract and by using cholestane as internal standard:

µg/g of each sterol in each sample = (Ai/Ax) X (Bi/Bx) X (Ci/Cx) where Ai = peak area of each sterol in each sample; Ax = peak area of each sterol standard in control; Bi = peak area of cholestane standard in control; Bx = peak area of cholestane in each sample; Ci = amount of each sterol standard in control; and Cx = amount of each sample used for analysis (AOAC, 1990). The relative amounts of each sterol in each meat sample were expressed on dry or wet matter basis, whereas each sterol in each culture from each meat sample was expressed as µg/ml of each culture.

Statistical analysis

The data were analyzed by using the SAS software system package utilizing the Analysis of Variance and Least Significant Differences Procedures. Differences between means

were tested at $P < 0.05$.

Flow chart of procedures used for the assessment of the viability of *E. coprostanoligenes* in fermented meat samples

0.5 g of each uninoculated or inoculated pork or mutton sausage was used to make a suspension in 1-1.5 ml PBS

↓

A 1 ml aliquot of suspension from each pork or mutton sausages was used to inoculate a 9 ml volume of media containing 0.2% cholesterol

↓

All cultures were incubated at 37° C for 96 hours

↓

All tubes were removed and examined for growth then vortexed for 15 seconds.

↓

A 1 ml aliquot of each culture was removed, extracted and analyzed for cholesterol and coprostanol concentrations

↓

Viability of *E. coprostanoligenes* in inoculated fermented pork or mutton sausages was assessed based on the presence of coprostanol concentration in culture from each fermented sausage when compared with controls

RESULTS AND DISCUSSION

The fresh raw ground pork and mutton samples were analyzed before and after the addition of the condiments and sausages were analyzed after fermentation for pH and moisture, total lipids, cholesterol and coprostanol content. The fermented pork samples were cooked; then samples were taken for analysis of pH and moisture, total lipids, cholesterol and coprostanol content. The cooked fermented pork samples then were vacuum-packed and stored at normal display temperature and after 1 month of storage samples were taken for analyses as mentioned above.

pH. The pH values of raw ground pork before and after seasoning (before fermentation) and pork sausage after fermentation for experiments 1 and 2 are shown in Table 2. The pH values of the raw ground pork before and after seasoning (before fermentation) in experiment 1 were not different from each other ($P > 0.05$), but differed from those of the other treatments ($P< 0.05$) (Table 2). The pH values of fermented pork sausages inoculated with the combination of 0.05 g of *E. coprostanoligenes* and *Lactobacillus* pellet in both experiments 1 and 2 were lower than those of the other treatments ($P<0.05$) (Table 2).

The pH values of pork sausages after cooking in experiment 2 and of cultures of suspensions from fermented pork sausages from experiments 1 and 2 are shown in Table 3. The pH values

Table 2. pH of raw ground pork before and after seasoning (before fermentation) and of pork sausages after 12 hours of fermentation at 37° C

Treatments	pH Values[a]	
	Experiment 1	Experiment 2
Before seasoning		
Raw ground pork	5.57±.01[b]	5.78[b]
Before fermentation		
Raw ground pork	5.60±.02[b]	5.91[c]
After fermentation		
Uninoculated pork (control)	5.80±.10[c]	5.51±.08[d]
Lactobacillus Pellet (0.05 g)	5.18±.79[d]	4.96±.13[e]
Lactobacillus + *E. coprostanoligenes* Pellet (0.05 g of each)	4.44±.02[e]	4.66±.13[f]
E. coprostanoligenes		
Pellet		
0.05 g	5.86±.03[c]	5.44±.10[g]
0.10 g	5.87±.03[c]	5.55±.06[h]
0.20 or 0.5 g	5.87±.01[c]	5.72±.01[b]
Liquid culture		
1.0 ml	5.78±.00[c]	5.71±.02[b]
2.0 ml	5.82±.04[c]	5.56±.04[h]
10.0 ml	5.78±.00[c]	5.56±.12[h]

[a]Values represent means and standard errors of triplicate or duplicate samples of each treatment. Treatments were not replicated.
[b-h]Means in the same column with the same superscript are not different ($P > 0.05$).

Table 3. pH of sausages after cooking in experiment 2 and of cultures from fermented pork sausages after 96 hours incubation at 37° C in experiments 1 and 2

	pH Values[a]		
	After cooking	Cultures	
Treatments	Expt. 2	Expt. 1	Expt. 2
Uninoculated pork (control)	6.12±.07[b]	6.44±.14[b]	6.72±.04[b]
Lactobacillus Pellet (0.05 g)	5.10±.16[c]	6.33±.17[c]	6.69±.24[b]
Lactobacillus + *E. coprostanoligenes* Pellet (0.05 g each)	4.77±.07[c]	6.27±3.17[d]	6.72±.08[b]
E. coprostanoligenes Pellet			
0.05 g	5.98±.07[d]	6.50±.01[e]	6.72±.04[b]
0.10 g	5.99±0 [d]	6.38±.01[b]	6.75±.17[b]
0.20 or 0.50 g	6.00±.06[d]	6.46±.14[b]	6.78±.11[b]
Liquid culture			
1.0 ml	6.01±.04[b]	6.44±.08[b]	6.77±.07[b]
2.0 ml	5.63±.57[e]	6.41±.02[b]	6.83±.09[b]
10.0 ml	6.02±.04[b]	6.57±.06[f]	6.78±.06[b]

[a]Values represent means and standard errors of triplicate samples of each treatment except samples treated with 0.5 g *E. coprostanoligenes* in duplicate. Treatments were not replicated.

[b-f]Means in the same column with the same superscript are not different ($P < 0.05$).

after cooking in experiment 2 of sausages inoculated with the combination of 0.05 g of *Lactobacillus* and *E. coprostanoligenes* pellet and with 0.05 g of *Lactobacillus* pellet were lower than the pH values of the uninoculated (control) sausages and those of the other treatments ($P < 0.05$)(Table 3). The pH values of cultures from sausages in experiment 1 inoculated with the combination of 0.05 g of *Lactobacillus* and 0.05 g *E. coprostanoligenes* pellet and from sausages inoculated with 0.05 of *Lactobacillus* were lower than those of the other treatments ($P < 0.05$). No differences ($P > 0.05$) were observed for pH values of cultures from fermented sausages in experiment 2.

The pH values of raw ground pork and mutton before and after seasoning (before fermentation) and sausages after fermentation, after cooking and after 1 month of storage after cooking in experiment 3 are shown in Table 4. Pork sausages inoculated with the combinations of 0.1 g, 0.25 g and 0.5 g of *E. coprostanoligenes* + 0.05 g of *Lactobacillus* pellet and with 0.05 g of *Lactobacillus* pellet pH values were lower than those of the other treatments ($P < 0.05$)(Table 4). The pH of cultures from uninoculated (control) pork sausages was lower than those of the other treatments ($P < 0.05$). The differences observed in pH occurred because no specific endpoint for pH was used in the experiment (Wu et al., 1991).

Lactobacillus and *E. coprostanoligenes* caused pH to

Table 4. pH values[a] of raw ground pork and mutton before and after seasoning and of pork and mutton sausages after 15 hours of fermentation at 37° C, after 1 month of storage and in cultures from sausages - Experiment three

Treatments	Before and After Fermentation	After Cooking	After Storage	Cultures
Before seasoning				
Raw ground				
Pork	5.80±.01[b]	----	----	----
Mutton	5.95±.00[c]	----	----	----
Before fermentation				
Raw ground				
Pork	5.92±.01[d]	----	----	----
Mutton	5.83±.01[b]	----	----	----
After fermentation				
Uninoculated (control)				
Pork	6.09±.00[e]	6.35±.05[b]	6.37±0[b]	6.56±.04[b]
Mutton	6.11±.00[f]	6.24±.01[c]	6.22±.01[c]	6.73±.002[c]
Lactobacillus Pellet (0.05 g) - Pork	5.07±.10[g]	5.11±.01[d]	4.97±.03[d]	6.74±.03[c]
Lactobacillus (0.05 g) + *E. coprostanoligenes*				
Pellet - Pork				
0.10 g	4.74±.01[h]	4.84±.01[e]	4.85±.02[e]	6.84±.02[d]
0.25 g	4.92±.03[f]	5.05±.02[f]	4.91±.02[f]	6.89±.01[d]
0.50 g	4.74±.05[h]	4.99±.02[g]	4.85±.03[g]	6.87±.01[e]
E. coprostanoligenes				
Pellet - Pork				
0.25 g	6.10±.02[f]	6.30±.02[h]	6.32±.03[h]	6.72±.05[c]
0.50 g	6.12±.01[i]	6.25±.02[i]	6.26±.00[i]	6.84±.02[d]
2.00 g	6.24±.01[j]	6.29±.02[h]	6.29±.00[j]	6.87±.04[d]
Pellet - Mutton				
0.5 g	6.04±.00[k]	6.22±.01[c]	6.15±.01[k]	6.99±.09[f]
1.0 g	6.09±.00[k]	6.23±.03[c]	6.20±.03[l]	6.76±.01[e]

[a]Values represent means and standard errors of triplicate samples of each treatment except samples treated with 2.0 g *E. coprostanoligenes* in duplicate. Treatments were not replicated.
[b-k]Means in the same column with the same superscript are not different ($P < 0.05$).
---- = Not evaluated

decrease, but when combinations of both bacteria were used the decrease was greater. The greater decrease in pH resulted because of the acid produced by *E. coprostanoligenes* (Freier et al., 1994). The pH of fresh mutton sausages stored at 2-4° C for 120 days did not differ ($P > 0.05$), but pH increased in sausages stored at 20-22° C for 120 days (Wu et al., 1991). The pH values observed in cultures from fermented pork sausages were more variable, which may have been caused by the amount of inoculum added to each culture tube.

Moisture content. Moisture content of raw ground pork before and after seasoning (before fermentation) and fermented pork sausages in experiments 1 and 2 are shown in Table 5. The moisture percentages in raw ground pork before and after seasoning in experiments 1 and 2 were not different from each other ($P > 0.05$). The moisture percentages of uninoculated (control) sausages after fermentation, sausages inoculated with 0.05 g of *Lactobacillus* pellet and with 0.5 g of *E. coprostanoligenes* pellet in experiment 1 were lower ($P < 0.05$) than the other treatments. No differences were observed between moisture percentages in experiment 1 in sausages inoculated with 0.1 and 0.2 g of *E. coprostanoligenes* pellet and 1 ml and 2 ml aliquots of liquid culture of *E. coprostanoligenes* and sausages inoculated with combination of 0.05 g of *Lactobacillus* and *E. coprostanoligenes* pellet ($P > 0.05$)(Table 5). In experiment 2, moisture percentages of the

Table 5. Moisture content of raw ground pork before and after seasoning (before fermentation) and of pork sausages after 12 hours of fermentation at 37° C

Treatments	Moisture Percentages[a]	
	Experiment 1	Experiment 2
Before Seasoning		
Raw ground pork	67.56[b]	66.06[b]
Before fermentation		
Raw ground pork	67.6±.08[b]	66.40[b]
After fermentation		
Uninoculated pork (control)	61.7±1.2[c]	64.7±1.1[c]
Lactobacillus Pellet (0.05 g)	61.7±1.2[c]	63.5±1.2[c]
Lactobacillus + *E. coprostanoligenes* Pellet (0.05 g of each)	65.0±1[b]	62.9±1.2[c]
E. coprostanoligenes		
Pellet		
0.05 g	61.7±1.2[c]	65.1±.36[c]
0.10 g	65.7±1.7[b]	65.0±1.6[c]
0.20 or 0.5 g	63.5±0.5[b]	64.8±.75[c]
Liquid culture		
1.0 ml	66.0±1[b]	63.4±1.2[c]
2.0 ml	67.0±1[b]	65.3±.3[b]
10.0 ml	68.0±1.7[d]	68.2±1.6[d]

[a]Values represent means and standard errors of triplicate or duplicate samples of each treatment. Treatments were not replicated.
[b-d]Means in the same column with the same superscript are not different ($P > 0.05$).

uninoculated (control) pork sausages and pork sausages treated with bacterial pellets and with 1.0 ml aliquot liquid of *E. coprostanoligenes* were lower than those of the other treatments ($P < 0.05$). Moisture percentage of sausages inoculated with 10 ml aliquot of liquid *E. coprostanoligenes* culture in experiments 1 and 2 were higher than those of the other treatments ($P< 0.05$) (Table 5).

Moisture content of raw ground pork and mutton before and after seasoning (before fermentation) and pork and mutton sausages after fermentation, after cooking and after 1 month of storage after cooking in experiment 3 are shown in Table 6. Moisture percentage of the raw ground pork before seasoning was higher than those of the other treatments ($P < 0.05$). Moisture percentages of uninoculated (control) pork and mutton sausages after fermentation and mutton sausages inoculated with 0.5 g and 1.0 g of *E. coprostanoligenes* pellets were lower than those the other treatments ($P < 0.05$)(Table 6). Moisture percentage observed of pork sausages inoculated with 0.25 g and 0.5 g of *E. coprostanoligenes* pellet after cooking and after 1 month of storage were higher than those of the other treatments ($P < 0.05$).

Moisture percentage was not consistently affected by the treatments and the variability may be as a result of the size of the ground meat and the type of starter culture used in the sausage production. Mutton sausage products made with Lactacel

Table 6. Moisture content of raw ground pork and mutton before and after seasoning (before fermentation) and of pork and mutton sausages after 15 hours of fermentation at 37° C, after cooking and after 1 month of storage after cooking - Experiment three

Treatments	Moisture Percentages[a]		
	Before and After Fermentation	After Cooking	After Storage
Before seasoning - Raw ground			
Pork	69.74±.72[b]	----	----
Mutton	61.93±.12[c]	----	----
Before fermentation - Raw ground			
Pork	64.55±.55[d]	----	----
Mutton	63.24±.23[d]	----	----
After fermentation			
Uninoculated (control)			
Pork	62.8±.92[c]	56.60± .52[b]	58.2±.09[b]
Mutton	60.1±.32[e]	54.90±1.3[c]	56.5±.08[c]
Lactobacillus Pellet (0.05 g) - Pork	63.22±.54[d]	56.07±1.6[b]	57.1±1.12[b]
Lactobacillus (0.05 g) + *E. coprostanoligenes*			
Pellet - Pork			
0.10 g	64.24±.04[f]	56.49±.22[b]	57.6±.3[d]
0.25 g	63.34±.28[g]	54.51±.66[b]	52.1±.46[e]
0.50 g	63.04±.46[d]	58.39±.37[c]	57.1±.42[b]
E. coprostanoligenes			
Pellet - Pork			
0.25 g	65.28±.53[h]	59.20±1.1[d]	59.7±.65[f]
0.50 g	63.60±.39[d]	59.19±.48[d]	61.8±.86[g]
2.00 g	64.35±.25[d]	57.79±.66[b]	58.5±.10[d]
Pellet - Mutton			
0.5 g	62.55±.17[c]	56.77±.79[b]	56.3±.29[c]
1.0 g	60.40±.52[i]	57.37±.63[b]	56.1±.61[c]

[a]Values represent means and standard errors of triplicate samples of each treatment except samples treated with 2.0 g *E. coprostanoligenes* in duplicate. Treatments were not replicated.

[b-i]Means in the same column with the same superscript are not different (P > 0.05). ---- = Not evaluated

75 starter culture had more ($P < 0.05$) moisture than sausage products made with *L. plantarium 27* (Wu et al., 1991).

Lipid content. Total lipid content of raw ground pork before and after seasoning (before fermentation) and fermented pork sausages in experiments 1 and 2 are shown in Table 7. Lipid percentages on both dry and wet matter basis observed in uninoculated (control) sausages (after fermentation) in experiment 1 were lower than those of the other treatments in both experiments ($P < 0.05$)(Table 7). Lipid percentages on both dry and wet matter basis in raw ground pork before and after seasoning in experiment 2 were not different from each other ($P > 0.05$), but were lower than those of uninoculated (control) fermented sausages and sausages inoculated with the bacterial pellets ($P < 0.05$)(Table 7).

Total lipid content of raw pork sausages after cooking and after 1 month of storage after cooking on wet matter basis in experiment 2 are shown in Table 8. Lipid percentages of uninoculated (control) sausages were not different ($P > 0.05$). Lipid percentages observed in sausages inoculated with 10 ml aliquot of liquid *E. coprostanoligenes* culture in both experiments were lower than those of the other treatments ($P < 0.05$)(Table 8).

Total lipid content of raw ground pork and mutton before and after seasoning (before fermentation) and pork and mutton sausages after fermentation and after cooking in experiment 3

Table 7. Total lipid content of raw ground pork before and after seasoning (before fermentation) and of pork sausages after 12 hours of fermentation at 37° C

	Lipid Percentages[a] on Dry and Wet Matter Basis			
	Experiment 1		Experiment 2	
Treatments	Dry	Wet	Dry	Wet
Before Seasoning				
Raw ground pork	NE[b]	NE	31.50±5.4[b]	10.7±1.8[b]
Before fermentation				
Raw ground pork	32.97±4.3[c]	10.7±1.0[c]	32.74±8.1[b]	11.0±2.8[b]
After fermentation				
Uninoculated pork (control)	22.06±4.3[d]	7.7±1.3[d]	35.10±8.4[c]	12.5±2.4[c]
Lactobacillus Pellet (0.05 g)	30.14±4.8[c]	10.6±1.4[c]	35.20±4.4[c]	12.9±1.1[c]
Lactobacillus + *E. coprostanoligenes* Pellet (0.05 g of each)	37.7±12.0[e]	13.3±4.7[e]	34.40±1.8[c]	12.8±1.2[c]
E. coprostanoligenes Pellet				
0.05 g	31.2± 3.7[c]	11.9±1.7[c]	40.7± 6.5[c]	14.2±2.4[d]
0.10 g	35.7± 8.1[e]	12.3±3.0[f]	45.3±11.3[d]	15.8±3.6[e]
0.20 or 0.5 g	26.9± 1.2[c]	9.8±0.2[c]	39.4±14.8[c]	14.6±3.7[d]
Liquid culture				
1.0 ml	35.5± 3.2[e]	12.1±1.2[c]	30.3± 6.5[b]	11.0±1.9[b]
2.0 ml	37.6±12.8[e]	12.3±1.0[f]	29.1± 1.7[b]	10.1±0.5[f]
10.0 ml	25.8± 4.3[c]	8.2±1.5[g]	35.2± 3.8[c]	11.2±2.0[b]

[a]Values represent means and standard errors of triplicate or duplicate samples of each treatment. Treatments were not replicated.
[b]NE = not evaluated
[c-g]Means in the same column with the same superscript are not different (P > 0.05).

Table 8. Total lipid content of fermented pork sausages after cooking and after 1 month of storage after cooking - Experiment two

Treatments	Lipid Percentages[a] on Wet Matter Basis	
	After cooking	After storage
Uninoculated pork (control)	13.3±1.9[b]	13.9±2[b]
Lactobacillus Pellet (0.05 g)	16.2±2.7[c]	15.5±1.4[c]
Lactobacillus + *E. coprostanoligenes* Pellet (0.05 g each)	16.8±3.2[c]	15.9±4.1[c]
E. coprostanoligenes Pellet		
0.05 g	14.2±1.2[b]	16.7± .4[c]
0.10 g	15.5± .8[d]	13.7±2.3[b]
0.50 g	13.3±2.1[b]	14.7±1.5[b]
Liquid culture		
1.0 ml	13.7±1.5[b]	13.7±2[b]
2.0 ml	12.2±1.8[e]	14.5± .7[b]
10.0 ml	11.6±1.8[f]	11.8± .6[d]

[a]Values represent means and standard errors of triplicate samples of each treatment except samples treated with 0.5 g *E. coprostanoligenes* in duplicate. Treatments were not replicated.
[b-f]Means in the same column with the same superscript are not different ($P > 0.05$).

are shown in Table 9. Lipid percentages of raw ground mutton and uninoculated (control) and treated mutton sausages on dry matter basis were higher than those of raw ground pork and pork sausages ($P < 0.05$). Lipid percentages in raw ground pork and mutton and sausages on wet matter basis and on both dry and wet matter basis after cooking were variable. After cooking, lipid percentages of uninoculated (control) pork sausages and pork sausages inoculated with 0.1 g of *E. coprostanoligenes* pellet on dry and wet matter basis were lower than those of the other treatments ($P < 0.05$)(Table 9).

Total lipid content of fermented pork and mutton sausages after 1 month of storage after cooking in experiment 3 are shown in Table 10. Lipid percentage of pork sausages inoculated with 0.1 g of *E. coprostanoligenes* pellet were lower than those of the other treatments on both dry and wet matter basis ($P < 0.05$) (Table 10). Lipid percentage of mutton sausages inoculated with 1.0 g *E. coprostanoligenes* pellet were higher than those of the other treatments on both dry and wet matter basis ($P < 0.05$)(Table 10).

The variability of lipid percentages in meat samples may have resulted from the conditions under which the sausages were processed, the size of the inoculum used and the interaction of the bacteria with the meat. Microorganisms can degrade meat fat by hydrolysis via a lipase and/or oxidation by oxidases (Bacus, 1984). *E. coprostanoligenes* possesses

Table 9. Total lipid content of raw ground pork and mutton before and after seasoning and of pork and mutton sausages after 15 hours of fermentation at 37° C and after cooking - Experiment three

	Lipid Percentages[a] on Wet and Dry Matter Basis			
	Before and After Fermentation		After Cooking	
Treatments	Dry	Wet	Dry	Wet
Before seasoning - Raw ground				
Pork	40.90±1.4[b]	12.58±.29[b]	----	----
Mutton	54.43±2.3[c]	20.71±.81[c]	----	----
Before fermentation - Raw ground				
Pork	35.58±3.5[d]	12.51±1.1[b]	----	----
Mutton	37.92±1.5[e]	13.54±.45[b]	----	----
After fermentation				
Uninoculated (control)				
Pork	35.68±1.6[d]	13.18±.37[b]	27.65±6.6[b]	12.03±1.3[b]
Mutton	47.55±2.3[f]	19.02±.96[d]	41.46±2.5[c]	18.79±.66[c]
Lactobacillus Pellet (0.05 g) - Pork	37.22±1.3[e]	13.65±.31[b]	31.72±2.1[c]	13.85±.80[d]
Lactobacillus (0.05 g) + *E. coprostanoligenes*				
Pellet - Pork				
0.10 g	39.00±3.6[b]	13.95±1.3[b]	28.11±1.9[b]	12.23±.82[b]
0.25 g	36.08±1.3[d]	13.22±.44[b]	33.84±3.0[c]	15.23±1.3[d]
0.50 g	37.84±1.1[e]	13.97±.31[b]	35.71±1.6[c]	14.83±.55[d]
E. coprostanoligenes				
Pellet - Pork				
0.25 g	33.88±1.0[d]	11.75±.36[e]	40.87±5.3[c]	16.69±2.1[d]
0.50 g	35.42±1.2[d]	12.92±.56[b]	34.75±1.2[c]	14.16±.38[d]
2.00 g	33.41±.58[d]	11.91±.08[e]	44.94±2.8[d]	19.03±2.1[c]
Pellet - Mutton				
0.5 g	41.62±1.5[b]	15.58±.51[f]	45.17±3.7[d]	19.46±1.5[e]
1.0 g	45.82±1.3[g]	18.14±.53[g]	42.20±1.5[d]	17.99±.68[c]

[a]Values represent means and standard errors of triplicate samples of each treatment except samples treated with 2.0 g *E. coprostanoligenes* in duplicate. Treatments were not replicated. ---- = Not evaluated

[b-g]Means in the same column with the same superscript are not different ($P > 0.05$).

Table 10. Total lipid content of fermented pork and mutton after 1 month of storage after cooking - Experiment three

Treatments	Lipid Percentages[a]	
	Dry	Wet
Uninoculated (control)		
Pork	33.45± .55[b]	13.98±.24[b]
Mutton	37.74±1.64[c]	16.60±.64[c]
Lactobacillus Pellet (0.05 g - Pork	30.57± .48[d]	13.15±.52[d]
Lactobacillus (0.05 g) + *E. coprostanoligenes* Pellet - Pork		
0.10 g	27.33±1.72[e]	11.62±.74[e]
0.25 g	35.28±1.28[f]	17.02±.73[c]
0.50 g	37.52±1.22[c]	16.08±.51[f]
E. coprostanoligenes Pellet - Pork		
0.25 g	31.44± .69[g]	13.10±.17[d]
0.50 g	36.78±1.17[f]	14.10±.70[g]
2.00 g	34.77± .99[b]	14.41±.37[g]
Pellet - Mutton		
0.50 g	35.48±5.2[f]	15.50±2.08[f]
1.00 g	39.12± .27[h]	17.17± .30[h]

[a]Values represent means and standard errors of triplicate samples of each treatment except samples treated with 1.0 g and 2.0 g *E. coprostanoligenes* in mutton and in pork which were in duplicate. Treatments were not replicated.
[b-h]Means in the same column with the same superscript are not different (P > 0.05).

phospholipase activity and requires lecithin for growth (Freier, 1991).

Cholesterol and coprostanol content. Cholesterol and coprostanol concentrations on dry and wet matter basis in raw ground pork after seasoning (before fermentation) and fermented pork sausages in experiment 1 are shown in Table 11. Cholesterol concentrations in raw ground pork before seasoning on dry and wet matter basis were lower than those of the other treatments ($P < 0.05$). Sausages inoculated with 0.1 g of *E. coprostanoligenes* pellet contained higher concentration of cholesterol on dry matter basis than those in the uninoculated (control) sausages and those of the other treatments ($P < 0.05$).

Cholesterol concentration on wet matter basis in sausages inoculated with 0.05 g and 0.1 g of *E. coprostanoligenes* pellet and with 0.05 g *Lactobacillus* pellet were not different from each other ($P > 0.05$), but contained higher cholesterol than those in the uninoculated (control) sausages and the other treatments ($P < 0.05$) (Table 11). No detectable amount of coprostanol was observed in any raw ground pork or fermented pork sausage samples.

Cholesterol and coprostanol concentrations in raw ground pork before and after seasoning (before fermentation) and pork sausages after fermentation on dry and wet matter basis in experiment 2 are shown in Table 12. Cholesterol concentration

Table 11. Cholesterol and coprostanol concentrations[a] in raw ground pork before and after seasoning (before fermentation) and of pork sausages after 12 hours of fermentation at 37° C - Experiment one

	Dry basis (µg/g)		Wet basis (µg/g)	
Treatments	Chol[b].	Cop[c].	Chol.	Cop.
Before fermentation				
Raw ground pork	329.1±46.3[e]	NDA[d]	124.2±24.8[e]	NDA
After fermentation				
Uninoculated pork (control)	685.3±32.1[f]	NDA	259.4±10.0[f]	NDA
Lactobacillus Pellet (0.05 g)	890.4±25.0[g]	NDA	349.6± 8.0[g]	NDA
Lactobacillus + *E. coprostanoligenes* Pellet (0.05 g of each)	727.8±33.5[h]	NDA	256.2±11.8[h]	NDA
E. coprostanoligenes				
Pellet				
0.05 g	904.6±37.4[g]	NDA	352.8±14.0[g]	NDA
0.10 g	968.4±21.3[i]	NDA	337.0± 7.4[g]	NDA
0.20 g	592.3±16.6[j]	NDA	214.0± 6.0[h]	NDA
Liquid culture				
1.0 ml	944.2±32.0[k]	NDA	324.8±11.0[i]	NDA
2.0 ml	846.1±23.4[l]	NDA	282.6± 7.8[h]	NDA
10.0 ml	746.3± 9.4[f]	NDA	238.8± 3.0[h]	NDA

[a]Values represent means and standard errors of triplicate or duplicate samples of each treatment. Treatments were not replicated.
[b]Chol = cholesterol
[c]Cop = coprostanol
[d]NDA = no detectable amount
[e-l]Means in the same column with the same superscript are not different (P > 0.05).

on dry matter basis in raw ground pork before seasoning was higher than those in the other treatments ($P < 0.05$). Cholesterol concentrations on wet matter basis in raw ground pork before seasoning and sausages inoculated with 0.5 g of *E. coprostanoligenes* pellet were not different from each other ($P > 0.05$), but were higher than those in the other treatments ($P < 0.05$) (Table 12). No detectable amount coprostanol was observed in any raw ground pork or fermented pork sausage samples.

Cholesterol and coprostanol concentrations detected in fermented pork sausages after cooking and after 1 month of storage in experiment 2 are shown in Table 13. The cholesterol concentrations observed after cooking on wet matter basis in sausages inoculated with the combination of 0.05 g of *Lactobacillus* and *E. coprostanoligenes* pellet was higher than those in the uninoculated (control) sausages and other treatments ($P < 0.05$) (Table 13). No detectable amount of coprostanol was observed in any uninoculated (control) or inoculated pork sausage.

After 1 month of storage after cooking, no differences were observed between cholesterol concentrations in the sausages ($P > 0.05$) (Table 13). No detectable amount of coprostanol was observed in any sausage sample.

Cholesterol and coprostanol concentration in cultures of suspensions from fermented pork sausages in experiments 1 and

Table 12. Cholesterol and coprostanol concentrations[a] in pork samples before and after 12 hours of fermentation at 37° C - Experiment two

	Dry basis (µg/g)		Wet basis (µg/g)	
Treatments	Chol[b].	Cop[c].	Chol.	Cop.
Before seasoning				
Raw ground pork	1188.20±150.0[e]	NDA[d]	404.0± 5.0[e]	NDA
Before Fermentation				
Raw ground pork	847.06± 50.0[f]	NDA	288.6±17.0[f]	NDA
After fermentation				
Uninoculated pork (control)	928.30± 13.9[g]	NDA	334.2± 5.0[g]	NDA
Lactobacillus Pellet (0.05 g)	867.80± 26.7[g]	NDA	312.4± 9.6[g]	NDA
Lactobacillus + *E. coprostanoligenes* Pellet (0.05 g each)	784.20± 30.5[e]	NDA	298.0±11.6[f]	NDA
E. coprostanoligenes				
Pellet				
0.05 g	991.1± 36.7[h]	NDA	356.8±13.2[g]	NDA
0.10 g	915.9± 17.1[g]	NDA	311.4± 5.8[g]	NDA
0.50 g	1061.1± 63.3[h]	NDA	382.0±22.8[e]	NDA
Liquid culture				
1.0 ml	936.7± 40.6[g]	NDA	337.2±14.6[g]	NDA
2.0 ml	959.4± 14.1[h]	NDA	326.2± 4.8[g]	NDA
10.0 ml	959.4± 22.5[g]	NDA	307.0± 7.2[f]	NDA

[a]Values represent means and standard errors of triplicate samples of each treatment except samples treated with 0.5 g *E. coprostanoligenes* in duplicate. Treatments were not replicated.
[b]Chol = cholesterol
[c]Cop = coprostanol
[d]NDA = no detectable amount
[e-h]Means in the same column with the same superscript are not different ($P > 0.05$).

Table 13. Cholesterol and coprostanol concentrations[a] in fermented pork sausages after cooking and after 1 month of storage after cooking - Experiment two

Treatments	After cooking		After Storage	
	Chol[b].	Cop.[c]	Chol.	Cop.
	Wet basis (µg/g)			
Uninoculated pork (control)	318.8± 5.8[e]	NDA[d]	448±17[e]	NDA
Lactobacillus Pellet (0.05 g)	326.0± 7.8[e]	NDA	431±26.4[e]	NDA
Lactobacillus + *E. coprostanoligenes* Pellet (0.05 g each)	396.0±28.4[f]	NDA	431.55.8[e]	NDA
E. coprostanoligenes Pellet				
0.05 g	293.0± 3.4[e]	NDA	372±12.4[e]	NDA
0.10 g	278.6±18.2[g]	NDA	363±10.6[e]	NDA
0.50 g	381.8± 3.4[e]	NDA	400± 3.6[e]	NDA
Liquid culture				
1.0 ml	290.4±16.8[g]	NDA	391± 2[e]	NDA
2.0 ml	320.2±13.6[e]	NDA	293.28.6[e]	NDA
10.0 ml	282.4± 3.8[g]	NDA	395± 8.2[e]	NDA

[a]Values represent means and standard errors of triplicate samples of each treatment except samples treated with 0.5 g *E. coprostanoligenes* in duplicate. Treatments were not replicated.
[b]Chol = cholesterol
[c]Cop = coprostanol
[d]NDA = no detectable amount
[e-g]Means in the same column with the same superscript are not different ($P > 0.05$)

2 are shown in Table 14. Cholesterol concentrations in cultures from uninoculated (control) sausages in experiment 1 was higher than those of the other treatments ($P < 0.05$). Cholesterol concentration observed in cultures from sausages inoculated with the combination of 0.05 g *Lactobacillus* and *E. coprostanoligenes* pellets and with 1.0 ml aliquot of liquid *E. coprostanoligenes* culture in experiment 2 were higher than those in the other treatments in both experiments ($P < 0.05$)(Table 14).

No detectable amount of coprostanol was observed in any cultures from any of the fermented pork sausages experiment 1. Coprostanol was detected only in cultures from sausages inoculated with 0.5 g of *E. coprostanoligenes* pellet in experiment 2. The detection of coprostanol in cultures from sausages inoculated with the 0.5 g of bacterial pellet showed that *E. coprostanoligenes* remain viable and maintained its ability to reduce cholesterol to coprostanol after fermentation.

Cholesterol and coprostanol concentrations in raw ground pork and mutton before seasoning, after seasoning (before fermentation) and pork and mutton sausages after fermentation in experiment 3 are shown in Table 15. Cholesterol concentrations observed in raw ground pork before and after seasoning (before fermentation), uninoculated (control) pork sausages after fermentation, pork sausages inoculated with

Table 14. Cholesterol and coprostanol concentrations[a] in cultures from pork sausages after fermentation - 96 hours of incubation at 37° C

Treatments	Experiment 1		Experiment 2	
	Chol[b].	Cop[c].	Chol.	Cop.
	(µg/ml)			
Uninoculated pork (control)	438.2±44.0[e]	NDA[d]	721.3±33.9[e]	NDA[d]
Lactobacillus Pellet (0.05 g)	226.3±20.4[f]	NDA	729.3±17.2[e]	NDA
Lactobacillus + *E. coprostanoligenes* Pellet (0.05 g each)	231.0±15.0[f]	NDA	828.0±90.5[f]	NDA
E. coprostanoligenes Pellet				
0.05 g	244.0± 6.4[g]	NDA	717.3±11.1[e]	NDA
0.10 g	378.9±50.6[g]	NDA	544.0±75.4[g]	NDA
0.20 or 0.5 g	429.5±54.0[h]	NDA	570.0±.93[h]	18.5±4.9
Liquid culture				
1.0 ml	230.2±11.9[f]	NDA	824.01±30.2[f]	NDA
2.0 ml	215.9±10.5[f]	NDA	773.32±40.6[e]	NDA
10.0 ml	286.8± 4.2[g]	NDA	761.28±19.3[e]	NDA

[a]Values represent means and standard errors of duplicate samples of each treatment. Treatments were not replicated.
[b]Chol = cholesterol
[c]Cop = coprostanol
[d]NDA = no detectable amount
[e-g]Means in the same column with the same superscript are not different ($P > 0.05$).

Table 15. Cholesterol and coprostanol concentrations[a] in pork and mutton before and after 15 hours of fermentation at 37° C - Experiment three

	Dry basis (µg/g)		Wet basis (µg/g)	
Treatments	Chol[b].	Cop[c].	Chol.	Cop.
Before seasoning				
Raw ground				
Pork	734.2±51.4[d]	3.5[d*]	222.8±10.8[d]	1.0[d*]
Mutton	1671.0±33.7[e]	1.8±.8[d**]	638.3± 9.5[e]	.7±.3[d**]
Before fermentation				
Raw ground				
Pork	518.1±20.9[d]	1.3±.1[d**]	203.3± 9.1[f]	.5±.0[e**]
Mutton	1933.3±47.3[f]	2.0±.1[d**]	687.8±20.3[g]	.7±.0[d**]
After fermentation				
Uninoculated (control)				
Pork	542.1± 27.0[d]	NDA[m]	198.4± 9.8[f]	NDA[m]
Mutton	2157.1±549.3[e]	3.6[d*]	868.4±210.1[h]	1.4[d*]
Lactobacillus Pellet (0.05 g) - Pork	844.3± 15.3[f]	1.7[d*]	366.9± 23.5[i]	.6[d*]
Lactobacillus (0.05 g) + *E. coprostanoligenes*				
Pellet - Pork				
0.10 g	504.6±26.8[g]	.9[d*]	180.0± 9.3[f]	.3[d*]
0.25 g	463.6±38.8[g]	1.7±.2[d***]	170.8±12.7[f]	.6±.1[e***]
0.50 g	492.1±22.1[g]	3.3±.6[d***]	181.4± 5.6[f]	1.2±.2[d***]
E. coprostanoligenes				
Pellet - Pork				
0.25 g	591.9±25.2[d]	2.5±.1[d**]	203.9± 7.7[f]	.8±.0[d**]
0.50 g	708.4±61.2[d]	3.1±.6[d**]	226.4±27.4[d]	1.1±.3[d**]
2.00 g	1272.1± .2[h]	11.8±.4[e**]	449.4± 1.9[j]	4.1±.2[d**]
Pellet - Mutton				
0.50 g	1414.1±27.6[i]	1.3±.5[d**]	532.1±12.7[k]	.7±.3[d**]
1.00 g	1535.0±75.0[i]	3.4±.2[d***]	609.2±23.3[l]	1.3±.1[d***]

[a]Values represent means and standard errors of triplicate samples of each treatment except samples treated with 2.0 g *E. coprostanoligenes* in duplicate. Treatments were not replicated. [b]Chol = cholesterol, [c]Cop = coprostanol. [d-l]Means in the same column with the same superscript are not different ($P < 0.05$). * = 1 positive, ** = 2 positive, ** = 3 positive, [m]NDA = no detectable amount.

0.25 g and 0.5 g of *E. coprostanoligenes* pellet on dry matter basis were not different ($P > 0.05$). Sausages inoculated with the combinations of 0.1 g, 0.25 g and 0.5 g of *E. coprostanoligenes* and 0.05 g of *Lactobacillus* pellets were not different from each other, but were lower than those of the other treatments ($P < 0.05$)(Table 15). Cholesterol concentrations observed in raw ground mutton and mutton sausages on dry and wet matter basis were higher than those of raw ground pork and pork sausages ($P < 0.05$).

Cholesterol concentrations on wet matter basis in raw ground pork, uninoculated (control) pork sausages, pork sausages inoculated with the combinations of 0.1 g, 0.25 g and 0.5 g of *E. coprostanoligenes* + 0.05 g of *Lactobacillus* pellet and with 0.25 g of *E. coprostanoligenes* pellet were not different from each other ($P > 0.05$), but they were lower than those of the other treatments ($P < 0.05$)(Table 15)(Figure 1).

Small amounts of coprostanol were detected in raw ground pork and mutton on dry and wet matter basis. No coprostanol was detected in the uninoculated (control) pork sausage after fermentation. The concentration of coprostanol observed on dry and wet matter basis in pork sausages inoculated with 2.0 g *E. coprostanoligenes* pellet were higher than the coprostanol concentrations detected in all other meat samples ($P < 0.05$) (Table 15). No differences were observed between coprostanol concentrations detected in the other meat samples ($P > 0.05$)

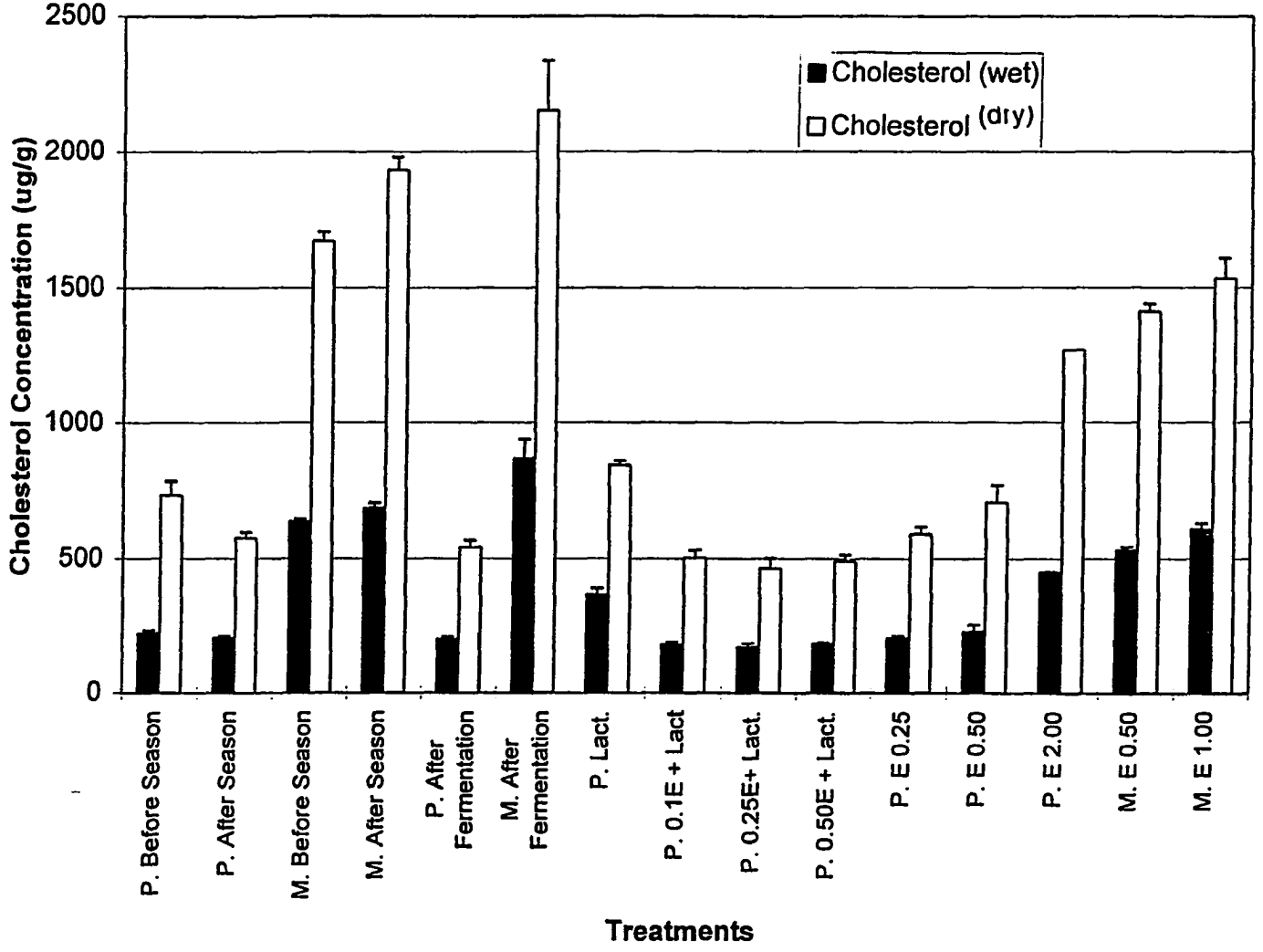

Figure 1. Cholesterol concentrations in meat samples before and after 15 hours of fermentation at 37° C, P = pork, M = mutton, Ferm = fermentation, Lact = *Lactobacillus*, E = *Eubacterium*, Season = seasoning. Values are expressed as treatment means ± SEM

(Table 15)(Figure 2).

Cholesterol and coprostanol concentrations in fermented pork and mutton sausages after cooking in experiment 3 are shown in Table 16. No differences were observed between cholesterol concentrations detected on dry and wet matter basis in the uninoculated (control) pork sausages and any of the inoculated pork sausages ($P > 0.05$)(Table 16). Cholesterol concentration in uninoculated mutton sausages was lower than those in mutton sausages inoculated with 0.5 g and 1.0 g of *E. coprostanoligenes* pellet ($P < 0.05$)(Table 16). Cholesterol concentrations observed on dry and wet matter basis in mutton sausages were higher than those of the pork sausages ($P < 0.05$)(Table 16)(Figure 3).

Pork sausages inoculated with 2.0 g *E. coprostanoligenes* pellet had the highest coprostanol concentration on dry and wet matter basis whereas no coprostanol was detected in the uninoculated (control) pork and mutton sausages, pork sausages inoculated with the combination of 0.5 g of *E. coprostanoligenes* + 0.05 g of *Lactobacillus* pellets and with 0.05 g of *Lactobacillus* pellet. No differences were observed on wet and dry between coprostanol concentrations detected in the sausages ($P > 0.05$)(Table 16)(Figure 4).

Cholesterol and coprostanol concentrations in fermented pork and mutton sausages after 1 month of storage after cooking showed that cholesterol was detected in all samples

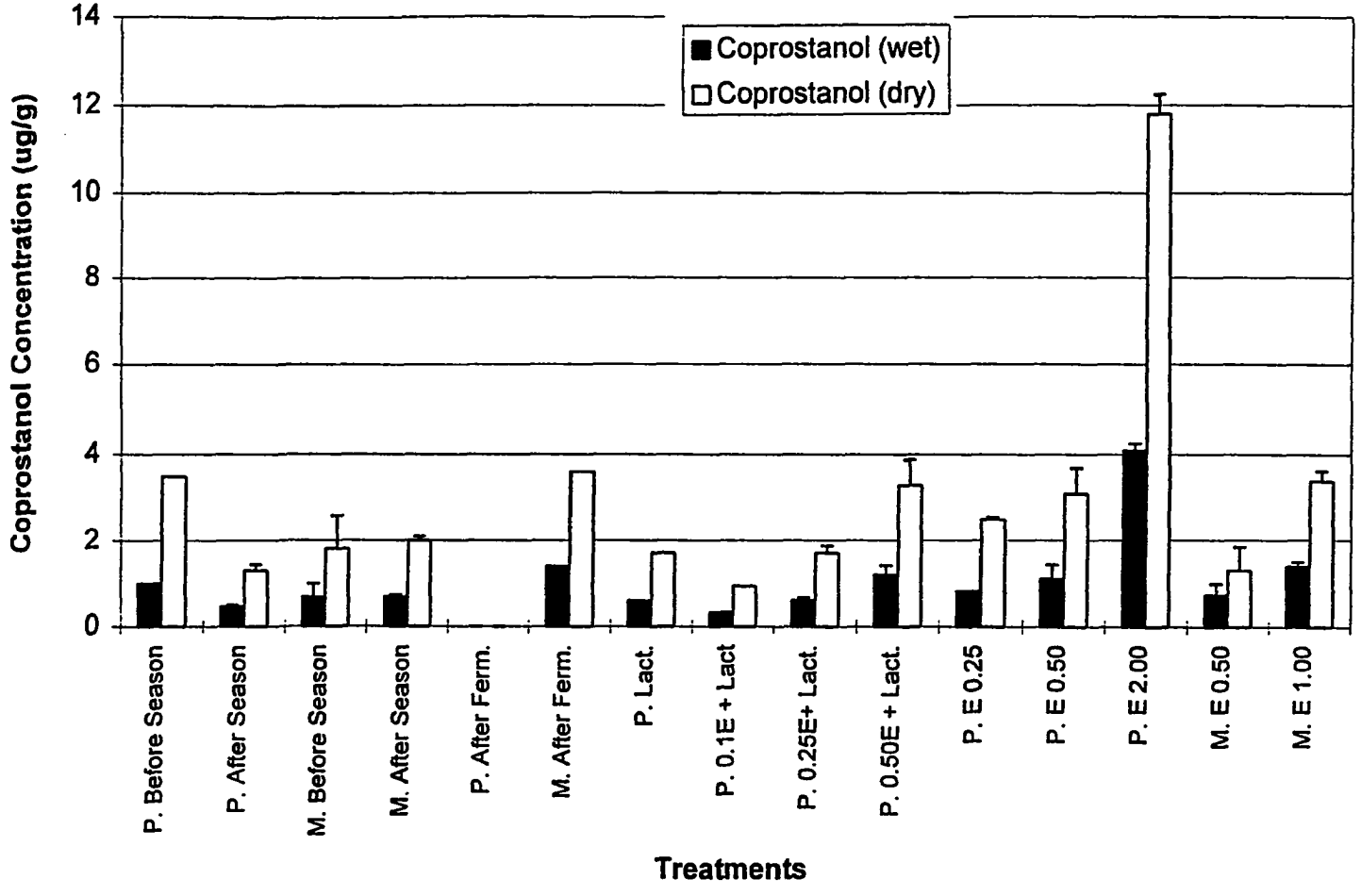

Figure 2. Coprostanol concentrations in meat samples before and after 15 hours of fermentation at 37° C, P = pork, M = mutton, Ferm = fermentation, Lact = *Lactobacillus*, E = *Eubacterium*, Season = seasoning. Values are expressed as treatment means ± SEM

Table 16. Cholesterol and coprostanol concentrations[a] in fermented pork and mutton sausages after cooking - Experiment three

Treatments	Dry basis (µg/g)		Wet basis (µg/g)	
	Chol[b].	Cop[c].	Chol.	Cop.
Uninoculated				
Pork	445.0±15.2[e]	NDA[d]	191.6± 5.7[e]	NDA
Mutton	1081.8±28.2[f]	NDA	493.5±17.9[f]	NDA
Lactobacillus Pellet (0.05 g) - Pork	527.2±31.6[e]	NDA	228.5± 7.8[e]	NDA
Lactobacillus (0.05 g)+ *E. coprostanoligenes* Pellet - Pork				
0.10 g	579.3±29.5[e]	3.3[e*]	252.3±13.0[e]	1.4[e*]
0.25 g	363.3±21.1[e]	3.2[e*]	159.7± 8.1[e]	1.5[e*]
0.50 g	630.3±46.1[e]	NDA[e]	263.1±21.4[e]	NDA
E. coprostanoligenes Pellet - Pork				
0.25 g	668.6±61.3[e]	4.0[e*]	285.6±20.3[e]	2.0[e*]
0.50 g	644.0±27.1[e]	6.2[e**]	265.7±13.6[e]	2.6[e**]
2.00 g	450.0±22.5[e]	8.2[e**]	189.0± 5.3[e]	3.5[e**]
Pellet - Mutton				
0.50 g	1257.3± 15.8[g]	5.0[e**]	544.7± 9.1[g]	2.1[e**]
1.00 g	1853.5±179.3[h]	7.8[e*]	320.2±13.6[h]	3.2[e*]

[a]Values represent means and standard errors of triplicate samples of each treatment except samples treated with 2.0 g *E. coprostanoligenes* in duplicate. Treatments were not replicated.
[b]Chol = cholesterol
[c]Cop = coprostanol
[d]NDA = no detectable amount
[e-h]Means in the same column with the same superscript are not different ($P < 0.05$).
* = 1 sample positive
** = 2 samples positive

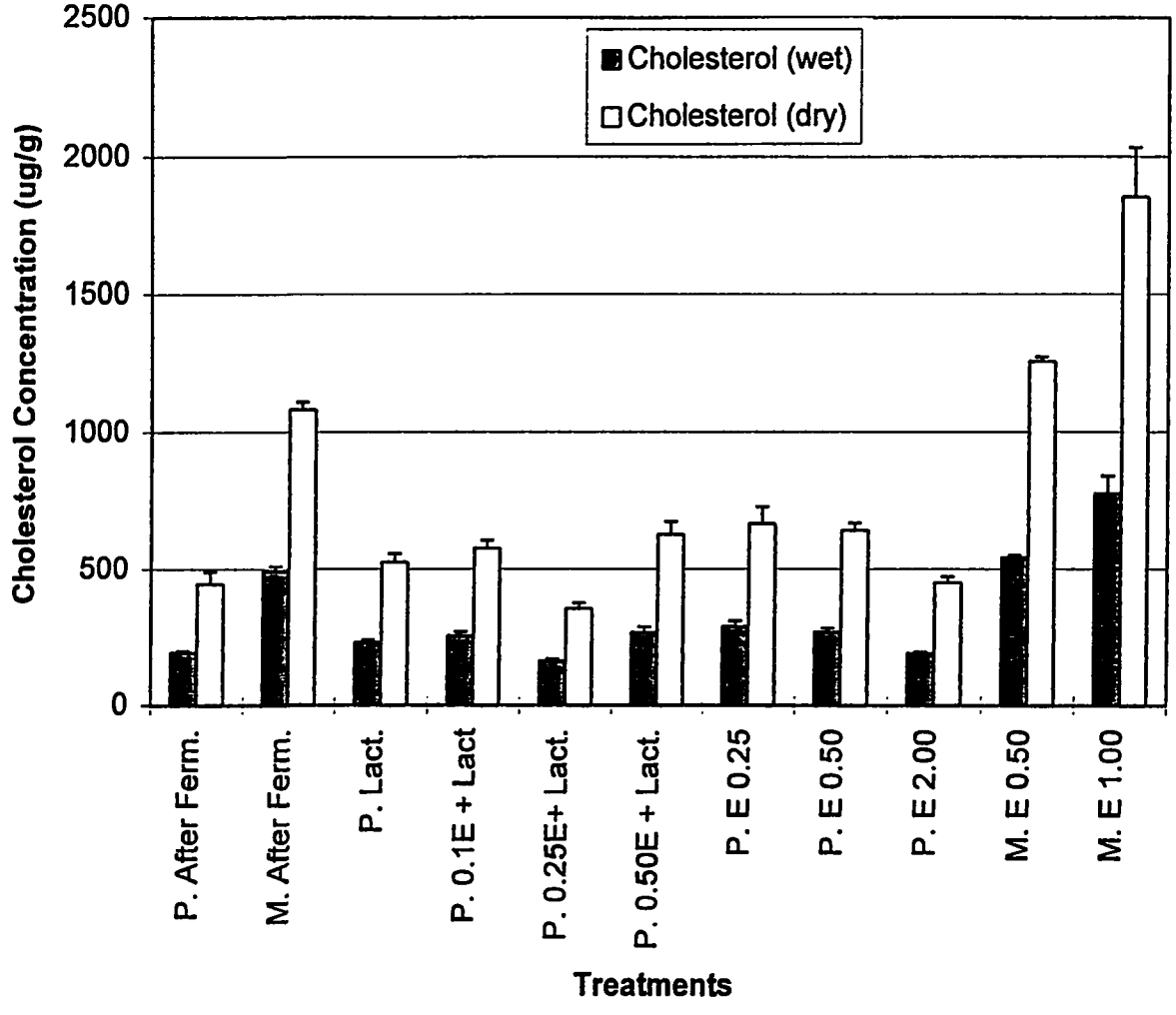

Figure 3. Cholesterol concentrations in fermented sausages after cooking at 68° C for 25 minutes, P = pork, M = mutton, Ferm = fermentation, Lact = *Lactobacillus*, E = *Eubacterium*. Values are expressed as treatment means ± SEM

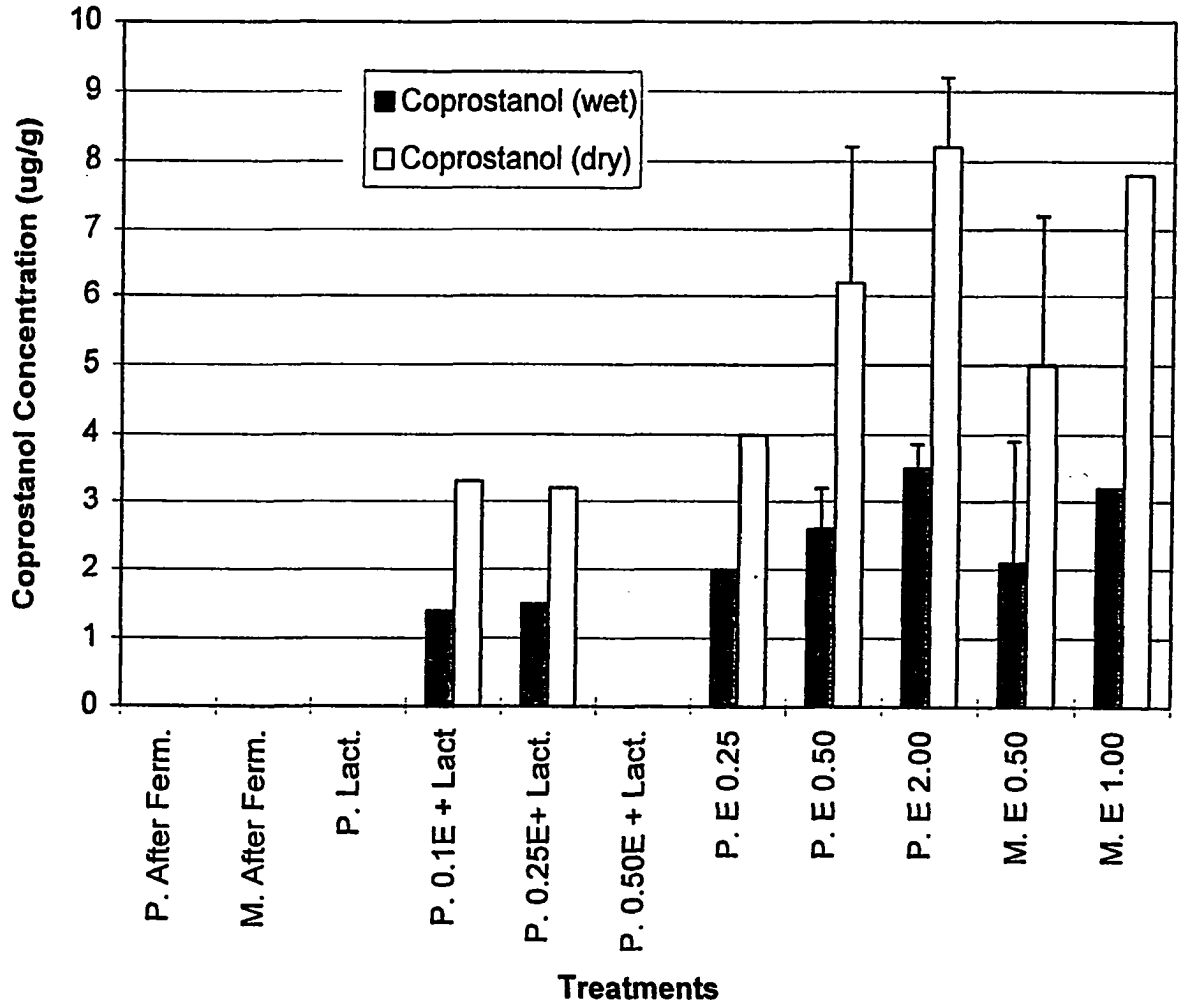

Figure 4. Coprostanol concentrations in fermented sausages after cooking at 68° C for 25 minutes, P = pork, M = mutton, Ferm = fermentation, Lact = *Lactobacillus*, E = *Eubacterium*. Values are expressed as treatment means ± SEM

but no coprostanol was detected in any pork or mutton sausage (data not shown).

Cholesterol and coprostanol concentrations observed in cultures of suspensions from fermented pork and mutton sausages after 96 hours incubation in experiment 3 are shown in Table 17. Cholesterol concentration detected in cultures from uninoculated (control) sausages were lower than those in cultures from the other sausages ($P < 0.05$). Cholesterol concentrations observed in cultures from pork sausages inoculated with 0.25 g and 0.5 g of *E. coprostanoligenes* pellet and with 0.25 g of *E. coprostanoligenes* + 0.05 g of *Lactobacillus* pellets and mutton sausages inoculated with 0.5 g of *E. coprostanoligenes* pellet were not different from each other ($P > 0.05$), but were higher than those of the other treatments ($P < 0.05$)(Table 17).

The highest coprostanol concentration was detected in cultures from pork sausages inoculated with 2.0 g of *E. coprostanoligenes* pellet whereas no coprostanol was detected in cultures from the uninoculated (control) pork and mutton sausages, pork sausages inoculated with the combinations of 0.1 g, 0.25 g and 0.5 g of *E. coprostanoligenes* + 0.05 g of *Lactobacillus* pellets. The coprostanol concentration detected in cultures from pork sausages inoculated with 2.0 g of *E. coprostanoligenes* pellet was higher than those of the other treatments ($P < 0.05$)(Table 17) (Figure 5). The

Table 17. Cholesterol and coprostanol concentrations[a] in cultures from pork sausages after fermentation - 96 hours of incubation at 37° C - Experiment three

Treatments	(µg/ml) Chol[b].	Cop[c].
Uninoculated (control)		
Pork	270.67±34.9[d]	NDA[d]
Mutton	439.32±72.3[e]	NDA
Lactobacillus Pellet (0.05 g) - Pork	465.81±25.2[e]	1.02±.21[e**]
Lactobacillus (0.05 g) + *E. coprostanoligenes* Pellet - Pork		
0.10 g	538.74± 46.5[e]	NDA
0.25 g	538.74±106.6[f]	NDA
0.50 g	214.68± 19.2[f]	NDA
E. coprostanoligenes Pellet - Pork		
0.25 g	547.16±45.3[f]	1.06±.40[e**]
0.50 g	537.79±51.5[d]	1.54±.64[e***]
2.00 g	452.93±27.3[e]	130.56±.93[f**]
Pellet - Mutton		
0.50 g	576.65±47.5[f]	2.31±.99[e**]
1.00 g	382.91±52.7[e]	.65[e*]

[a]Values represent means and standard errors of triplicate samples of each treatment except sample treated with 2.0 g *E. coprostanoligenes* in duplicate. Treatments were not replicated.
[b]Chol = cholesterol
[c]Cop = coprostanol
[d]NDA = no detectable amount
[e-h]Means in the same column with the same superscript are not different (P< 0.05).
* = 1 sample positive
** = 2 samples positive
*** = 3 samples positive

coprostanol concentrations detected in the other cultures not different from each other ($P > 0.05$).

The detection of coprostanol in meat samples and in cultures inoculated with suspensions from the fermented sausages showed that *E. coprostanoligenes* remained viable while converting cholesterol in pork and mutton during fermentation and maintained its ability to reduce cholesterol to coprostanol after fermentation when added to media containing cholesterol.

Cholesterol concentration in the fermented sausages and cultures showed much variability which could have resulted from several factors: the size of the ground meat used in the production of the sausages, the amount of cholesterol present in the meats, the type of starter culture used and the interactions of the bacteria with the condiments added to the meats and with the meats themselves and the conditions under which the sausages were produced.

Mutton sausages made from *Pediococcus acidilactici* H had lower ($P < 0.05$) percent retention of cholesterol during processing and storage than those made from Lactacel 75 and from *Lactobacillus plantarum* 27 (Wu et al., 1991). Lipolytic microorganisms degrade meat fats by hydrolysis via a lipase and/or oxidation by oxidases liberating fatty acids (Bacus, 1984). *E. coprostanoligenes* possesses phospholipase activity and requires lecithin for growth. Lecithin was hydrolyzed

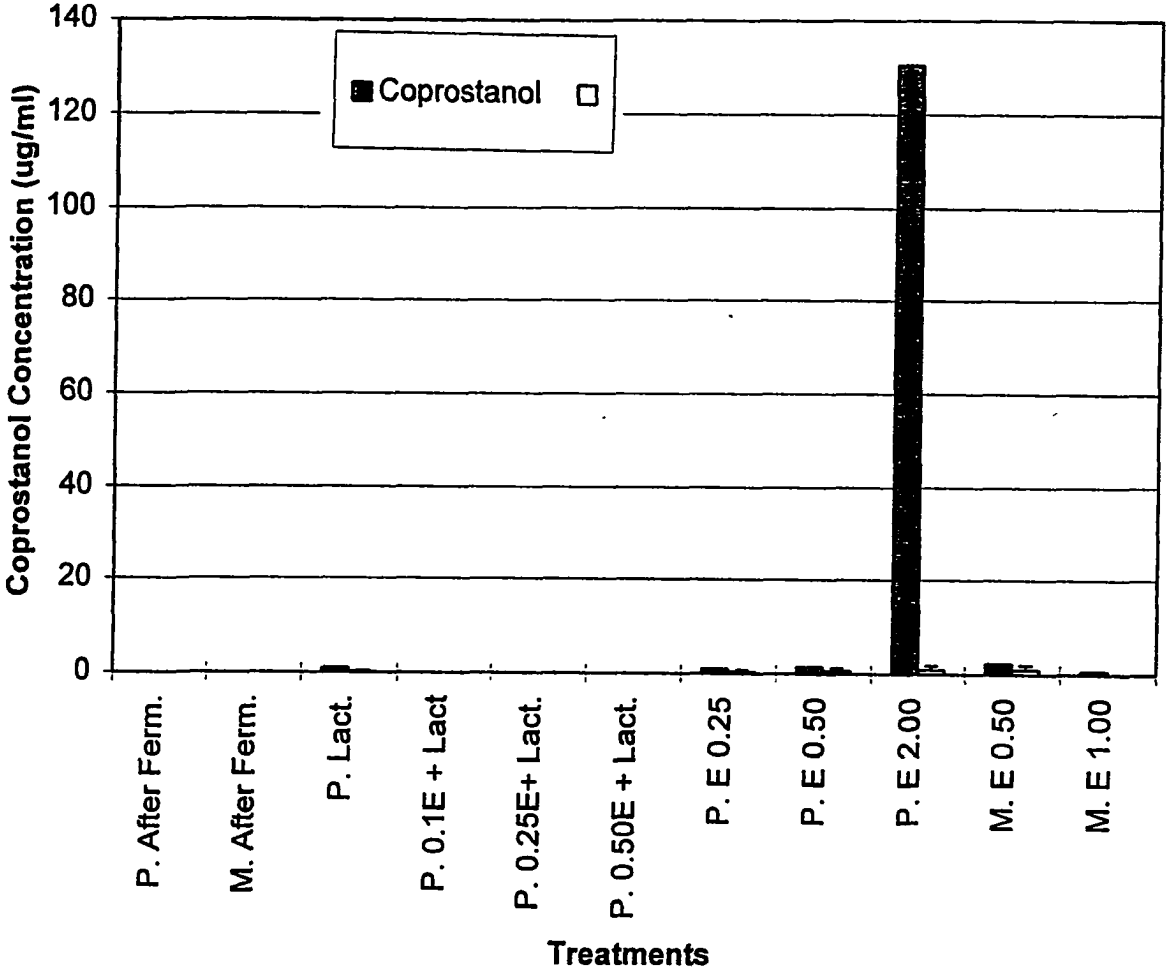

Figure 5. Coprostanol concentrations in cultures inoculated with suspensions from fermented sausages, cultures were incubated for 96 hours at 37° C, P = pork, M = mutton, Ferm = fermentation, Lact = *Lactobacillus*, *E* = *Eubacterium*. Values are expressed as treatment means ± SEM

because free fatty acids were detected in the media, but lecithin was not detected (Freier, 1991, Frier et al., 1994). *E. coprostanoligenes* may cause hydrolysis of cholesteryl esters in the meat liberating cholesterol and free fatty acids.

Fatty acids liberated by the interactions of the bacteria with the meats may be inhibitory to the microorganisms (Bacus, 1984) and may have affected the ability of the bacteria to reduce cholesterol. *E. coprostanoligenes* did not convert cholesterol to coprostanol when fatty acids were added to media containing cholesterol and no lecithin (Freier, 1991). Cholesterol content was greater ($P < 0.05$) in high-temperature-stored products, but no differences ($P < 0.05$) was observed for fresh cooked sausages and 120-day sausages stored at 2-4° or 20-22° C (Wu et al., 1991).

CONCLUSIONS

The results suggest that addition of *Lactobacillus* and *E. coprostanoligenes* caused pH to decrease in fermented pork and mutton sausages. When combinations of *Lactobacillus* + *E. coprostanoligenes* were used, lower ($P < 0.05$) pH values were observed when compared with sausages inoculated with *Lactobacillus*. Moisture, total lipid composition and cholesterol content were not consistently affected by the

bacterial treatments. Small amounts of coprostanol were detected in raw ground pork and mutton before fermentation. After fermentation no coprostanol was detected in the uninoculated (control) pork sausages. The highest concentration of coprostanol was detected in pork sausages inoculated with 2.0 g of *E. coprostanoligenes* pellet. Both *Lactobacillus* and *E. coprostanoligenes* produced coprostanol in the pork and mutton during fermentation.

No coprostanol was detected in cultures from uninoculated control) pork and mutton sausages and pork sausages inoculated with the various combinations of *Lactobacillus* + *E. coprostanoligenes* after 96 hours of incubation. Culture from sausages inoculated with 2.0 g of *E. coprostanoligenes* produced higher ($P < 0.05$) concentration of coprostanol after 96 hours of incubation. The detection of coprostanol in cultures from fermented sausages and in cultures from fermented sausages confirmed that *E. coprostanoligenes* will remain viable during fermentation of pork and mutton sausages while converting cholesterol to coprostanol and will maintain its ability to reduce cholesterol to coprostanol during and after fermentation. *E. coprostanoligenes* may be used to decrease the cholesterol content of fermented meats.

Additional studies are needed to determine the effectiveness of *E. coprostanoligenes* to decrease the cholesterol content of meats. These studies have identified

several areas of specific research needs:

1. Determination of the maximal growth phase of *E. coprostanoligenes*,
2. Determination of the optimal pH and temperature required for growth of the bacteria in meats,
3. Determination of the most suitable size of ground meat needed for adequate growth of the bacteria and reduction of cholesterol to coprostanol during fermentation,
4. Determination of the adequate amount of inoculum of *E. coprostanoligenes* needed for growth of the bacteria in meats and conversion of cholesterol to coprostanol during fermentation,
5. Determination of the consistency of the ground meat required for adequate growth of the bacteria and reduction of cholesterol to coprostanol during fermentation,
7. Determination of the optimal protein, fat and moisture content of meat required for adequate growth of the bacteria and reduction of cholesterol to coprostanol during fermentation and
8. Determination of the optimal time required for adequate growth of the bacteria in meat and reduction of cholesterol to coprostanol during fermentation.

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GENERAL CONCLUSIONS

In the first paper of this dissertation, New Zealand White adult male rabbits were used to investigate the potential for the use of *E. coprostanoligenes*, a cholesterol-reducing bacteria, to decrease plasma cholesterol concentration as a model for human hypercholesterolemia. The objectives were; 1) To feed a cholesterol-enriched diet to make the rabbits hypercholesterolemic and to evaluate the effects of dietary administered *E. coprostanoligenes* on (a) rabbit plasma cholesterol concentration (b) rabbit health and (c) fecal coprostanol and cholesterol ratios.

Oral administration of *E. coprostanoligenes* decreased cholesterol concentration in rabbits receiving a 0.1% cholesterol diet. Changes in plasma cholesterol concentration from day 0-77 were significantly lower ($P < 0.01$) in rabbits fed the 0.1% cholesterol + *E. coprostanoligenes* in 2% milk diet than plasma cholesterol concentration in rabbits fed the 0.1% cholesterol + boiled *E. coprostanoligenes* in 2% milk diet.

Several liver enzyme activities and major metabolites in plasma evaluated were not influenced by the bacterial treatments. The results suggest that incorporation of *E. coprostanoligenes* in milk and feeding to New Zealand White adult male rabbits receiving a 0.1% cholesterol-enriched has

been shown to be nontoxic and nonpathogenic to rabbits as no changes were observed in plasma enzymes GOT, GPT, LDH and ALP activities and major metabolites bilirubin, uric acid and total protein concentrations during the bacterial feeding when compared with the activities and concentrations at the initiation of the bacterial feeding.

The results further suggest that *E. coprostanoligenes* since seemed to be nontoxic and nonpathogenic in rabbits, it may be safe for human consumption. Milk may be used as a medium to deliver a cholesterol-reducing microbe to decrease plasma cholesterol concentration in humans. The decrease in plasma cholesterol concentrations observed in hypercholesterolemic rabbits fed *E. coprostanoligenes* may have important impacts on human health and welfare.

In the second paper the ability of *Eubacterium coprostanoligenes* to decrease cholesterol content of fermented pork and mutton sausages was evaluated as a model for decreasing the cholesterol content of foods. The viability of *E. coprostanoligenes* in the fermented sausages was evaluated by analysis of the concentrations of cholesterol and coprostanol in fermented pork and mutton sausages and in cultures inoculated with suspensions from the fermented sausages. Moisture, total lipid content and cholesterol content were not consistently affected by the bacterial treatments. The highest concentration of coprostanol was

detected in the 100 g pork sausages inoculated with the 2.0 g of *E. coprostanoligenes* pellet. Both *Lactobacillus* and *E. coprostanoligenes* produced coprostanol in the ground pork during fermentation.

Sausages inoculated with various combinations of *Lactobacillus* + *E. coprostanoligenes* did not produce coprostanol in cultures containing 0.2% cholesterol after 96 hours of incubation at 37° C. Cultures from fermented sausages inoculated with 2.0 g of *E. coprostanoligenes* also produced significantly higher ($P < 0.05$) concentration of coprostanol than all other treatments after 96 hours of incubation at 37° C. The detection of coprostanol in cultures from the fermented sausages confirmed that *E. coprostanoligenes* will remain viable during fermentation and will maintain its ability to reduce cholesterol to coprostanol during and after fermentation. *E. coprostanoligenes* may be used to decrease the cholesterol content of fermented meats because coprostanol was produced.

The results obtained from these two studies suggest that there are some potential for the development and application of technologies to use *E. coprostanoligenes* to treat human hypercholesterolemia. There is potential for prevention of hypercholesterolemia by utilization of this bacteria and/or cholesterol reductase enzyme isolated from this bacteria in food processing which should result in the lowering of the

cholesterol content in foods. This technology should result in the availability of foods with a more favorable cholesterol content and maintaining the nutrition, aesthetic, palatability and other organoleptic qualities. Informed consumers who are concerned about the composition of their diet and patients with hypercholesterolemia could now benefit from this technology.

The potential exist for treatment of hypercholesterolemia by utilization of this bacteria and/or cholesterol reductase enzyme if research is continued and the results should further indicate the safety of the bacteria in humans. Hypercholesterolemic human subjects may benefit from ingestion of an effective dose of the bacteria and/or enzyme in a capsule form which would facilitate passage into the lower gastrointestinal tract or consumption of an effective dose of the bacteria and/or enzymes in commonly consumed foods.

Additional studies are needed to determine the effectiveness of *E. coprostanoligenes* to decrease plasma cholesterol concentration of model animals and then humans and the cholesterol content of meats and other animal-derived foods. The following are some future research considerations:

1. Determination of the minimum effective dose of *E. coprostanoligenes* needed to achieve a decrease in plasma cholesterol concentration.
2. Determination of the by-products of metabolism and evaluate

for toxicity.

3. Isolation and purification of cholesterol reductase enzyme produced by *E. coprostanoligenes*.
4. Physical and chemical characterization of cholesterol reductase.
5. Determination of the mechanism(s) of cholesterol reductase reduction of cholesterol to coprostanol.
6. Identification and isolation of the gene(s) involved in the regulation of cholesterol reductase activity.
7. Determination of the interaction of *E. coprostanoligenes* with the condiments used in sausage production.
8. Determination of the most suitable conditions and dose needed for *E. coprostanoligenes* to grow in meats and other animal-derived products and to effectively decrease their cholesterol content.

APPENDIX A BODY WEIGHTS OF RABBITS

Table 1. Body weight of rabbits fed a commercial chow followed by a 0.1% cholesterol-enriched diet and *Eubacterium coprostanoligenes* in 2% fat milk for 21 days

	Body Weight[a] (kg)										
	Periods of Experiment (days)										
Groups	0	7	14	28	35	42	49	56	63	70	77
1	3.8 ±.08	4.0 ±.08	3.9 ±.08	4.1 ±.08	4.0 ±.05	4.0 ±.08	4.1 ±.1	4.0 ±.03	4.1 ±.1	4.1 ±.05	4.0 ±.1
2	3.8 ±.02	3.8 ±.02	3.9 ±.03	3.9 ±.03	3.8 ±.03	3.9 ±.03	3.9 ±.03	3.8 ±.05	3.9 ±.05	3.7 ±.05	3.7 ±.03
3	3.8 ±.03	3.8 ±.03	3.8 ±.03	3.7 ±.03	3.7 ±.03	3.8 ±.03	3.8 ±.03	3.8 ±.03	3.8 ±.03	3.8 ±.04	3.8 ±.05

[a]Values represent means and standard errors of body weight per group of rabbits corresponding to days of experiment. Bacterial feeding was initiated on day 28 and terminated on day 49. Feeding of the 0.1% cholesterol-enriched diet was continued until day 77. Groups: 1 = No cholesterol (control), N = 4; 2 = 0.1% cholesterol + boiled *E. coprostanoligenes* in 2% fat milk, N = 6; 3 = 0.1% cholesterol + *E. coprostanoligenes* in 2% fat milk, N = 8.

Table 2. Body weight of rabbits fed a commercial chow followed by a 0.1% cholesterol-enriched diet and *Eubacterium coprostanoligenes* in 2% fat milk for 21 days.

Body Weight[a] (kg)						
	Days of Experiment					
Groups	108	111	118	125	132	137
1	4.1 ±.05	4.0 ±.08	4.0 ±.1	4.1 ±.03	4.1 ±.1	4.0 ±.1
2	3.9 ±.03	3.8 ±.03	3.9 ±.03	3.8 ±.05	3.7 ±.05	3.7 ±.03
3	3.7 ±.03	3.7 ±.03	3.8 ±.03	3.8 ±.03	3.8 ±.03	3.8 ±.05

[a]Values represent means and standard errors of body weight per group of rabbits corresponding to days of experiment. Bacterial feeding was initiated on day 28 and terminated on day 49. Feeding of the 0.1% cholesterol-enriched diet was continued until day 77. Regular chow was fed from day 78 to day 108, then followed by 0.1% cholesterol diet from day 109 to day 137.
Groups: 1 = No cholesterol (control), N = 4; 2 = 0.1% cholesterol + boiled *E. coprostanoligenes* in 2% fat milk, N = 6; 3 = 0.1% cholesterol + *E. coprostanoligenes* in 2% fat milk, N = 8.

APPENDIX B PLASMA CHOLESTEROL CONCENTRATION IN RABBITS

Group 1. No cholesterol (control)

	Plasma Cholesterol Concentration (g/100 ml)								
	Days of Experiment								
Rabbit No.	0	7	14	28	35	42	49	56	63
2145	16.4	12.0	10.9	6.7	9.6	12.7	8.4	12.4	13.0
2149	19.0	22.0	21.1	14.6	18.1	18.1	16.1	17.2	19.9
2168	12.6	15.2	14.0	12.3	12.5	14.7	13.3	16.2	14.6
2169	30.8	36.3	23.5	26.5	23.9	20.4	21.3	19.4	28.4
Group 2.	Boiled *Eubacterium* in 2% fat milk								
2144	120.3	135.8	119.8	118.4	214.2	247.4	305.9	293.9	275.1
2147	42.4	64.6	48.7	39.8	91.8	96.7	114.9	95.0	90.2
2150	180.2	252.0	194.7	270.0	258.7	253.6	297.7	345.5	274.3
2153	116.5	137.5	104.0	162.8	236.7	259.8	309.3	264.5	266.7
2161	38.0	45.3	50.8	55.5	88.8	75.2	95.3	125.4	91.8
2163	82.2	129.0	138.9	138.9	137.4	140.1	111.3	103.7	123.1
Group 3.	*Eubacterium* in 2 % fat milk								
2142	85.2	78.2	85.2	83.8	109.2	116.8	97.1	84.3	66.7
2148	27.7	31.4	32.8	20.8	33.3	42.6	58.1	52.1	55.0
2151	316.8	290.1	289.9	198.6	251.2	264.6	310.7	321.6	280.5
2155	162.2	173.2	166.2	202.1	257.4	287.3	318.1	273.0	320.4
2156	61.7	57.3	55.3	55.7	53.3	67.4	74.1	64.7	64.7
2157	114.4	123.6	96.1	120.7	167.5	175.9	242.4	186.1	213.9
2158	323.6	327.3	296.5	306.5	330.8	288.8	310.1	288.9	282.1
2164	49.3	59.6	44.5	51.9	64.9	86.0	93.5	72.2	74.1

Group 1. No cholesterol (control)

	Plasma Cholesterol Concentration (g/100 ml)							
	Days of Experiment							
Rabbit No.	70	77	108	111	118	125	132	137
2145	13.5	11.7	15.9	14.0	18.6	19.7	19.1	16.6
2149	23.4	23.9	19.1	15.4	20.5	23.9	26.3	32.6
2168	14.3	15.5	10.9	9.2	16.6	21.8	19.2	14.8
2169	22.4	16.9	12.7	16.0	27.5	31.8	37.9	25.8
Group 2.	Boiled *Eubacterium* in 2% fat milk							
2144	276.4	309.8	80.6	99.6	114.9	127.7	128.7	111.9
2147	90.7	73.1	13.3	16.8	29.3	34.9	59.1	33.6
2150	229.1	306.8	241.2	264.1	256.4	282.0	291.3	290.8
2153	281.3	284.6	71.8	86.8	137.6	168.5	169.3	208.0
2161	87.0	123.0	7.8	5.9	24.1	31.9	21.4	12.6
2163	112.8	131.5	238.7	261.7	253.1	299.8	290.1	285.1
Group 3.	*Eubacterium* in 2 % fat milk							
2142	75.3	83.9	17.3	27.2	45.2	55.0	52.6	34.4
2148	52.2	60.5	12.2	14.5	26.5	37.4	38.2	20.9
2151	281.3	309.8	273.7	232.3	171.2	159.1	162.9	158.5
2155	258.4	283.3	111.1	139.3	209.9	206.7	255.7	265.2
2156	64.3	55.4	16.8	30.7	44.6	50.6	68.4	28.6
2157	206.4	222.5	54.1	72.7	115.3	147.8	154.9	132.5
2158	283.3	310.5	275.2	267.4	252.5	249.8	300.4	297.8
2164	89.2	76.3	12.2	19.9	44.6	57.6	77.3	41.7

APPENDIX C PLASMA ENZYME ACTIVITIES AND MAJOR METABOLITE CONCENTRATIONS IN RABBITS

Group 1. No cholesterol (control)

	Day 28 of Experiment						
	GOT	GPT	LDH	ALP	Total Protein	Bilirubin	Uric acid
Rabbit No.	(IU/L)				(g/100 ml)	(mg/100 ml)	
2145	13	14	287	0	5.4	0.1	0.1
2149	17	45	242	0	5.4	0.1	0.1
2168	10	24	365	0	5.7	0	0
2169	7	23	0	0	5.3	0.1	0.2
Group 2. Boiled *Eubacterium* in 2% fat milk							
2144	13	22	189	0	1	0	0.3
2150	12	28	211	2	6.0	3.5	0.9
2153	9	36	172	4	6.1	0	0.2
2163	21	34	249	4	5.6	0	0.4
Group 3. *Eubacterium* in 2 % fat milk							
2151	9	22	187	5	6.8	0.1	0.5
2155	0	19	283	2	5.1	0	0.7
2157	12	22	165	0	6.2	0.3	0
2158	22	21	223	16	7.5	0	6.5

Group 1. No cholesterol (control)							
	Day 42 of Experiment						
	GOT	GPT	LDH	ALP	Total Protein	Bilirubin	Uric acid
Rabbit No.	(IU/L)				(g/100 ml)	(mg/100 ml)	
2145	3	17	7	0	5.1	0.1	0.2
2149	10	55	73	0	5.0	0.1	0.2
2168	12	22	288	0	5.7	0	0.1
2169	10	24	0	0	5.4	0.1	0.1
Group 2. Boiled *Eubacterium* in 2% fat milk							
2144	25	29	287	0	0	0	0.5
2150	12	26	283	3	0.4	0.1	0.7
2153	16	29	248	9	7.2	0	1.0
2163	13	32	167	1	5.3	0.1	0.2
Group 3. *Eubacterium* in 2 % fat milk							
2151	17	26	326	7	6.3	0	0.8
2155	10	25	58	1	5.3	0	1.1
2157	13	24	321	1	5.7	0	0.3
2158	13	15	254	2	5.8	0.2	0.9

Group 1. No cholesterol (control)

	Day 56 of Experiment						
	GOT	GPT	LDH	ALP	Total Protein	Bilirubin	Uric acid
Rabbit No.	(IU/L)				(g/100 ml)	(mg/100 ml)	
2145	8	21	293	1	5.2	0.1	0.1
2149	20	71	314	0	5.1	0.1	0
2168	10	21	288	0	4.9	0	0
2169	0	1	281	0	5.6	0.1	0
Group 2. Boiled *Eubacterium* in 2% fat milk							
2144	41	11	47	2	5.7	0	0.4
2150	13	22	357	3	0	0.3	0.7
2153	9	28	327	5	6.0	0	0
2163	17	37	247	0	5.3	0.1	0.1
Group 3. *Eubacterium* in 2 % fat milk							
2151	0	1	396	3	5.9	1.2	0.3
2155	16	28	350	0	0	0.1	0
2157	16	27	334	0	0	0	0.1
2158	1	1	305	0	5.4	0	0.2

APPENDIX D FECAL STEROLS AND BILE ACID CONCENTRATIONS

Group 1. No cholesterol (control)

Fecal Sterol Concentration (ug/g)		
	Day 0 of Experiment	
Rabbit No.	Cholesterol	Coprostanol
2145	6.23	0
2149	30.58	0
2168	0	0
2169	8.23	0
Group 2.	Boiled *Eubacterium* in 2% fat milk	
2144	31.67	0
2147	27.33	0
2150	25.29	0
2153	27.33	0
2161	0	0
2163	24.55	0
Group 3.	*Eubacterium* in 2 % fat milk	
2142	43.83	8.99
2148	10.49	0
2151	27.50	0
2155	74.17	4.07
2156	34.78	0
2157	16.56	0
2158	41.30	0
2164	20.30	0

Group 1. No cholesterol (control)

Fecal Sterol Concentration (ug/g)		
	Day 28 of Experiment	
Rabbit No.	Cholesterol	Coprostanol
2145	129.5	187.2
2149	113.6	100.0
2168	177.2	181.3
2169	132.6	39.5
Group 2.	Boiled *Eubacterium* in 2% fat milk	
2144	154.8	170.7
2147	195.0	179.1
2150	464.2	274.3
2153	181.9	138.2
2161	179.8	274.3
2163	302.9	128.1
Group 3.	*Eubacterium* in 2 % fat milk	
2142	172.7	137.7
2148	368.0	238.7
2151	163.4	59.6
2155	351.2	210.3
2156	124.3	89.3
2157	154.1	3.7
2158	273.3	94.3
2164	256.2	122.8

Group 1. No cholesterol (control)

Fecal Sterol Concentration (ug/g)		
Day 42 of Experiment		
Rabbit No.	Cholesterol	Coprostanol
2145	114.9	0
2149	139.8	0
2168	67.6	71.2
2169	95.0	47.5
Group 2.	Boiled *Eubacterium* in 2% fat milk	
2144	446.6	113.4
2147	294.9	14.5
2150	524.2	211.4
2153	349.7	72.6
2161	482.5	188.5
2163	785.7	162.9
Group 3.	*Eubacterium* in 2 % fat milk	
2142	289.3	0
2148	506.4	0
2151	289.4	0
2155	400.6	56.2
2156	229.5	63.8
2157	451.8	108.2
2158	712.9	31.2
2164	225.5	49.1

Group 1. No cholesterol (control)

Fecal Sterol Concentration (ug/g)		
Day 56 of Experiment		
Rabbit No.	Cholesterol	Coprostanol
2145	107.7	147.0
2149	123.2	179.2
216	87.3	159.3
2169	150.5	28.3
Group 2.	Boiled *Eubacterium* in 2% fat milk	
2144	0	0
2147	109.1	60.7
2150	172.8	128.5
2153	155.2	169.3
2161	130.8	134.9
2163	271.8	120.0
Group 3.	*Eubacterium* in 2 % fat milk	
2142	48.5	22.4
2148	72.4	43.0
2151	227.2	73.4
2155	224.6	84.0
2156	120.9	39.9
2157	154.1	86.8
2158	184.3	80.4
2164	80.3	40.5

Group 1. No cholesterol (control)

	Fecal Sterol Concentration (ug/g)	
	Day 77 of Experiment	
Rabbit No.	Cholesterol	Coprostanol
2145	37.7	0
2149	27.1	13.8
2168	27.2	29.0
2169	140.1	31.2
Group 2.	Boiled *Eubacterium* in 2% fat milk	
2144	80.5	31.3
2147	101.0	65.3
2150	66.1	0
2153	51.5	15.1
2161	119.9	88.0
2163	73.7	23.5
Group 3.	*Eubacterium* in 2 % fat milk	
2142	156.1	142.8
2148	101.6	42.3
2151	434.2	512.6
2155	99.3	133.9
2156	33.2	14.6
2157	80.0	15.6
2158	349.9	80.1
2164	126.0	47.5

Group 1. No cholesterol (control)		
	Fecal Sterol Concentration (ug/g)	
	Day 132 of Experiment	
Rabbit No.	Cholesterol	Coprostanol
2145	18:0	14.1
2149	0	0
2168	0	0
2169	105.8	57.0
Group 2. Boiled *Eubacterium* in 2% fat milk		
2144	137.7	25.2
2147	15.5	66.1
2150	213.7	222.0
2153	201.1	28.1
2161	292.3	216.8
2163	291.9	328.3
Group 3. *Eubacterium* in 2 % fat milk		
2142	138.4	30.8
2148	210.5	57.9
2151	65.0	59.0
2155	380.8	92.8
2156	179.1	8.2
2157	240.4	52.2
2158	125.3	78.0
2164	190.4	105.2

Group 1. No cholesterol (control)		
	Fecal Sterol Concentration (ug/g)	
	Day 137 of Experiment	
Rabbit No.	Cholesterol	Coprostanol
2145	133.2	94.6
2149	50.5	0
2168	56.6	31.2
2169	65.3	0
Group 2. Boiled *Eubacterium* in 2% fat milk		
2144	14.5	16.0
2147	89.8	63.4
2150	255.9	219.3
2153	54.6	10.5
2161	164.7	106.7
2163	357.5	428.5
Group 3. *Eubacterium* in 2 % fat milk		
2142	145.8	37.2
2148	110.3	98.5
2151	139.3	52.5
2155	139.2	54.2
2156	59.3	0
2157	221.9	103.0
2158	59.8	0
2164	55.1	5.4

Group 1. No cholesterol (control)

	Fecal Sterol Concentration (ug/g)	
	Day 0 of Experiment	
Rabbit No.	ß-sitosterol	Deoxycholic acid
2145	2.0	35.2
2149	1.1	27.9
2168	0	36.9
2169	97.7	27.9
Group 2.	Boiled *Eubacterium* in 2% fat milk	
2144	0	47.9
2147	5.1	41.3
2150	3.9	24.3
2153	67.1	24.5
2161	0	4.8
2163	323.5	43.8
Group 3.	*Eubacterium* in 2 % fat milk	
2142	2.4	77.0
2148	0	20.7
2151	2.1	23.2
2155	87.0	77.9
2156	54.4	47.3
2157	0	24.6
2158	124.8	87.0
2164	4.0	22.5

Group 1. No cholesterol (control)

Fecal Sterol Concentration (ug/g)		
	Day 28 of Experiment	
Rabbit No.	ß-sitosterol	Deoxycholic acid
2145	92.5	7.5
2149	1625.3	358.3
2168	1.2	25.3
2169	2573.7	503.0
Group 2.	Boiled *Eubacterium* in 2% fat milk	
2144	9.1	72.9
2147	1230.4	284.5
2150	81.7	90.5
2153	2305.8	301.5
2161	1309.9	213.6
2163	3281.5	410.0
Group 3.	*Eubacterium* in 2 % fat milk	
2142	1322.0	243.6
2148	384.0	82.6
2151	1231.4	269.2
2155	396.2	82.9
2156	1227.4	250.7
2157	1683.2	262.6
2158	2785.7	453.8
2164	2930.7	435.6

Group 1. No cholesterol (control)

Fecal Sterol Concentration (ug/g)		
Day 42 of Experiment		
Rabbit No.	ß-sitosterol	Deoxycholic acid
2145	1770.9	235.6
2149	2390.8	289.7
2168	1279.3	208.0
2169	1313.9	201.2
Group 2.	Boiled *Eubacterium* in 2% fat milk	
2144	1906.8	250.8
2147	1593.6	226.6
2150	2540.7	260.4
2153	2226.7	228.0
2161	1719.6	220.6
2163	3700.0	447.0
Group 3.	*Eubacterium* in 2 % fat milk	
2142	1548.3	441.4
2148	2018.0	311.6
2151	1128.3	183.3
2155	1884.6	254.6
2156	1050.5	125.8
2157	2934.8	340.0
2158	2307.7	293.1
2164	1250.3	195.0

Group 1. No cholesterol (control)

	Fecal Sterol Concentration (ug/g)	
	Day 56 of Experiment	
Rabbit No.	ß-sitosterol	Deoxycholic acid
2145	1928.4	249.7
2149	2176.0	276.8
2168	1793.3	275.1
2169	2278.7	313.6
Group 2.	Boiled *Eubacterium* in 2% fat milk	
2144	0	0
2147	863.8	128.8
2150	2361.5	228.4
2153	2118.5	231.3
2161	1385.3	172.1
2163	2070.6	314.1
Group 3.	*Eubacterium* in 2 % fat milk	
2142	562.1	75.9
2148	593.1	123.6
2151	1635.0	153.4
2155	2520.0	295.2
2156	1232.3	172.3
2157	1505.7	231.9
2158	1970.2	217.9
2164	1030.2	102.7

Group 1. No cholesterol (control)		
	Fecal Sterol Concentration (ug/g)	
	Day 77 of Experiment	
Rabbit No.	ß-sitosterol	Deoxycholic acid
2145	1907.0	225.6
2149	1011.5	147.6
2168	860.2	196.9
2169	3326.9	356.1
Group 2. Boiled *Eubacterium* in 2% fat milk		
2144	934.1	151.1
2147	1728.3	284.4
2150	1617.5	177.7
2153	1239.3	163.3
2161	2276.6	340.4
2163	1559.5	221.2
Group 3. *Eubacterium* in 2 % fat milk		
2142	4684.6	584.3
2148	2513.9	400.0
2151	2954.5	373.8
2155	2179.2	313.9
2156	554.6	138.1
2157	1532.5	298.7
2158	4772.6	763.6
2164	3440.8	434.6

Group 1. No cholesterol (control)

	Fecal Sterol Concentration (ug/g)	
	Day 132 of Experiment	
Rabbit No.	ß-sitosterol	Deoxycholic acid
2145	0	80.7
2149	84.0	110.8
2168	7.0	0
2169	704.8	404.5
Group 2.	Boiled *Eubacterium* in 2% fat milk	
2144	540.4	342.0
2147	623.6	478.1
2150	506.7	307.0
2153	881.7	453.9
2161	731.5	437.6
2163	873.3	344.0
Group 3.	*Eubacterium* in 2 % fat milk	
2142	551.2	316.6
2148	1027.5	540.9
2151	319.4	156.1
2155	1693.2	973.4
2156	902.9	543.8
2157	1069.3	601.6
2158	227.7	251.7
2164	760.0	452.0

Group 1. No cholesterol (control)

	Fecal Sterol Concentration (ug/g)	
	Day 137 of Experiment	
Rabbit No.	ß-sitosterol	Deoxycholic acid
2145	1664.2	535.3
2149	1566.1	401.8
2168	1613.7	117.9
2169	1965.7	481.5
Group 2.	Boiled *Eubacterium* in 2% fat milk	
2144	103.9	42.6
2147	495.4	293.5
2150	1918.2	469.3
2153	1146.9	151.4
2161	1372.2	502.8
2163	1683.9	432.3
Group 3.	*Eubacterium* in 2 % fat milk	
2142	1597.4	499.9
2148	534.7	328.8
2151	917.9	482.1
2155	830.8	546.2
2156	425.5	236.4
2157	1725.9	740.7
2158	2051.1	232.3
2164	362.2	177.8

APPENDIX E DATA FROM MEAT FROM EXPERIMENT ONE

Table 1. Wet and dry weight of samples and weight of total lipid (g), cholesterol and coprostanol (µg) in pork before and after fermentation samples - Experiment one

Treatments	Weights[a] (g) Wet	Dry	Lipid	(µg) Chol.	Cop.
Before fermentation Raw ground pork	.501 ±.00001	.1621 ±.0001	.0535 ±.002	.0621 ±.06	NDA[b]
After fermentation					
Uninoculated pork (control)	.501 ±.0002	.1759 ±.001	.0388 ±.003	1.297 ±.05	NDA
Lactobacillus Pellet (0.05 g)	.501 ±.0002	.1753 ±.002	.0599 ±.009	1.749 ±.04	NDA
Lactobacillus + *E. coprostanoligenes* Pellet (0.05 g of each)	.502 ±.001	.1757 ±.003	.0624 ±.01	1.281 ±.06	NDA
E. coprostanoligenes Pellet					
0.05 g	.501 ±.003	.1920 ±.03	.0487 ±.001	1.764 ±.07	NDA
0.10 g	.506 ±.0002	.1743 ±.003	.0608 ±.006	1.683 ±.04	NDA
0.20 g	.495 ±.0002	.1810 ±.002	.0620 ±.02	1.07 ±.03	NDA
Liquid culture					
1.0 ml	.503 ±.002	.1713 ±.001	.0412 ±.007	1.624 ±.06	NDA
2.0 ml	.504 ±.003	.1667 ±.002	.0665 ±.02	1.413 ±.04	NDA
10.0 ml	.502 ±.001	.1603 ±..003	.0529 ±.009	1.195 ±.02	NDA

[a]Values represent means and standard errors of triplicate or duplicate samples of each treatment. Treatments were not replicated.
[b]NDA = no detectable amount

APPENDIX F DATA FROM MEAT FROM EXPERIMENT TWO

Table 1. Wet and dry weights of samples, and weight of total lipid (g), cholesterol and coprostanol (μg) of raw pork before seasoning, after seasoning (before fermentation) and pork fermented sausages - Experiment two

Treatments	Sample Weights (g)[a]				
	(g)			(μg)	
	Wet	Dry	Lipid	Chol.	Cop.
Before seasoning Raw ground pork	.504 ±0	.1698 ±.0	.0535 ±.003	2.02 ±.003	NDA[b]
Before fermentation Raw ground pork	.502 ±.01	.1683 ±.01	.0551 ±.003	1.44 ±.09	NDA
After fermentation Uninoculated pork (control)	.504 ±.003	.1780 ±.005	.0631 ±.003	1.65 ±.03	NDA
Lactobacillus Pellet (0.05 g)	.502 ±.002	.1836 ±.007	.0645 ±.003	1.56 ±.04	NDA
Lactobacillus + E. coprostanoligenes Pellet (0.05 of each)	.503 ±.005	.1869 ±.02	.0642 ±.003	1.48 ±.06	NDA
E. coprostanoligenes Pellet					
0.05 g	.502 ±.003	.1751 ±.003	.0714 ±.003	1.78 ±.11	NDA
0.10 g	.501 ±.002	.1755 ±.01	.0791 ±.006	1.54 ±.03	NDA
0.50 g	.499 ±.004	.1759 ±.004	.0690 ±.006	1.89 ±.07	NDA
Liquid culture					
1.0 ml	.501 ±.002	.1835 ±.007	.0553 ±.003	1.67 ±.07	NDA
2.0 ml	.503 ±.002	.1742 ±.002	.0507 ±.0006	1.61 ±.02	NDA
10.0 ml	.501 ±.002	.1596 ±.01	.0564 ±.003	1.52 ±.04	NDA

[a]Values represent means and standard errors of triplicate samples of each treatment except 0.5 g *E. coprostanoligenes* in duplicates.
[b]NDA = no detectable amount

Table 2. Wet and dry weights of samples, and weight of total lipid (g), cholesterol and coprostanol (μg) of fermented pork sausages after cooking - Experiment two

Treatments	Sample Weights (g)[a] (g) Wet	Dry	Lipid	(μg) Chol.	Cop.
Uninoculated pork (control)	.500 ±.003	.1768 ±.001	.0666 ±.003	1.625 ±.03	NDA[b]
Lactobacillus Pellet (0.05 g)	.500 ±.001	.1828 ±.002	.0810 ±.003	1.630 ±.04	NDA
Lactobacillus + *E. coprostanoligenes* Pellet (0.05 g each)	.501 ±.003	.1862 ±.006	.0842 ±.006	1.980 ±.14	NDA
E. coprostanoligenes Pellet					
0.05 g	.500 ±.003	.1746 ±.001	.0711 ±.003	1.464 ±.02	NDA
0.10 g	.500 ±.002	.1752 ±.003	.0768 ±.002	1.40 ±.09	NDA
0.50 g	.502 ±.0002	.1770 ±.003	.0666 ±.003	1.909 ±.16	NDA
Liquid culture					
1.0 ml	.501 ±.00003	.1835 ±.003	.0687 ±.003	1.452 ±.08	NDA
2.0 ml	.504 ±.0003	.1746 ±.0006	.0613 ±.003	1.601 ±.07	NDA
10.0 ml	.503 ±.001	.1602 ±..003	.0583 ±.003	1.412 ±.02	NDA

[a]Values represent means and standard errors of triplicate samples of each treatment except 0.5 g *E. coprostanoligenes* in duplicates.
[b]NDA = no detectable amount

Table 3. Wet and dry weights of samples, and weight of total lipid (g), cholesterol and coprostanol (µg) of fermented pork sausages after 1 month's storage after cooking - Experiment two

Treatments	Sample Weights[a] (g) Wet	Dry	Lipid	(µg) Chol.	Cop.
Uninoculated pork (control)	.503 ±.001	.1777 ±.002	.0699 ±.003	2.240 ±.09	NDA[b]
Lactobacillus Pellet (0.05 g)	.502 ±.0006	.1887 ±.001	.0778 ±.003	2.153 ±.13	NDA
Lactobacillus + *E. coprostanoligenes* Pellet (0.05 g each)	.501 ±.003	.1859 ±.006	.0793 ±.006	2.154 ±.28	NDA
E. coprostanoligenes Pellet					
0.05 g	.502 ±.0006	.1753 ±.0006	.0838 ±.0006	1.786 ±.06	NDA
0.10 g	.502 ±.001	.1758 ±.003	.0690 ±.003	1.814 ±.05	NDA
0.50 g	.502 ±0	.1770 ±.003	.0732 ±.003	2.001 ±.02	NDA
Liquid culture					
1.0 ml	.503 ±.0006	.1806 ±.001	.0686 ±.003	1.954 ±.01	NDA
2.0 ml	.502 ±.0006	.1740 ±.0006	.0729 ±.001	1.467 ±.14	NDA
10.0 ml	.504 ±.0006	.1604 ±.003	.0597 ±.001	1.976 ±.04	NDA

[a]Values represent means and standard errors of triplicate samples of each treatment except 0.5 g *E. coprostanoligenes* in duplicates.
[b]NDA = no detectable amount

APPENDIX G DATA FROM MEAT FROM EXPERIMENT THREE

Table 1. Wet and dry weights of samples and weight of total lipid (g), cholesterol and coprostanol (μg) of raw ground pork and mutton before fermentation and pork and mutton sausages after 15 hours fermentation - Experiment three

Treatments	Sample Weights[a] (g) Wet	Dry	Lipid	(μg) Chol[b].	Cop[c].
Before seasoning (Raw ground)					
Pork	.500	.1542	.0629	1.37	.006[e]
	±0	±.004	±.001	±.07	
Mutton	.500	.1904	.1036	5.03	.005[f]
	±0	±.0006	±.004	±.08	±.0003
Before fermentation (Raw ground)					
Pork	.500	.1772	.0625	1.75	.004[f]
	±0	±.003	±.005	±.08	±.0003
Mutton	.500	.1788	.0677	5.27	.005[f]
	±0	±.001	±.002	±.08	±.0003
After fermentation					
Uninoculated (control)					
Pork	.503	.1867	.0662	1.49	NDA[d]
	±.001	±.005	±.002	±.07	
Mutton	.503	.2013	.0957	5.41	.009[e]
	±.0007	±.002	±.005	±.43	
Lactobacillus	.500	.1838	.0682	1.59	.003[e]
Pellet (0.05 g)	±.0003	±.003	±.002	±.04	
Pork					
Lactobacillus (0.05 g) + *E. coprostanoligenes*					
Pellet - Pork					
0.10 g	.504	.1802	.0703	1.57	.003[e]
	±.0003	±.0001	±.006	±.08	
0.25 g	.507	.1858	.0670	1.61	.006
	±.001	±.002	±.002	±.12	±.0005
0.50 g	.504	.1864	.0705	1.72	.012
	±.002	±.003	±.002	±.06	±.002
E. coprostanoligenes					
Pellet - Pork					
0.25 g	.498	.1729	.0585	1.70	.007[f]
	±.001	±.003	±.002	±.06	±.00004
0.50 g	.499	.1815	.0644	1.65	.007[f]
	±.0007	±.002	±.003	±.12	±.002
2.00 g	.502	.1788	.0597	2.09	.019[f]
	±.004	±.003	±.0001	±.03	±.001

Treatments	Sample Weights[a] (g) Wet	Dry	Lipid	(μg) Chol.	Cop.
Pellet - Mutton					
0.5 g	.507	.1897	.0789	5.41	.007[f]
	±.001	±.0008	±.003	±.14	±.003
1.0 g	.502	.1987	.0911	5.05	.011
	±.002	±.002	±.003	±.20	±.0008

[a]Values represent means and standard errors of triplicate samples of each treatment except 2.0 g *E. coprostanoligenes* which was in duplicate.
[b]Chol = cholesterol
[c]Cop = coprostanol
[d]NDA = no detectable amount
[e] = 1 sample positive
[f] = 2 samples positive

Table 2. Wet and dry weights of samples and weight of total lipid (g), cholesterol and coprostanol (µg) of fermented pork and mutton sausages after cooking - Experiment three

Treatments	Sample Weights[a] (g) Wet	Dry	Lipid	(µg) Chol[b].	Cop[c].
Uninoculated (control)					
Pork	.501	.2176	.0603	1.44	NDA[d]
	±.001	±.002	±.006	±.04	
Mutton	.503	.2296	.0945	3.71	NDA
	±.002	±.006	±.004	±.13	
Lactobacillus Pellet (0.05 g) Pork	.500	.2194	.0691	1.55	NDA
	±.001	±.008	±.004	±.06	
Lactobacillus (0.05 g) + *E. coprostanoligenes* Pellet - Pork					
0.10 g	.500	.2177	.0611	1.82	.0101[e]
	±.001	±.0009	±.004	±.09	
0.25 g	.502	.2265	.0765	1.52	.0143[e]
	±.001	±.004	±.007	±.08	
0.50 g	.501	.2086	.0744	1.33	NDA
	±.002	±.001	±.003	±.11	
E. coprostanoligenes Pellet - Pork					
0.25 g	.503	.2052	.0840	1.40	.0096[e]
	±.002	±.006	±.01	±.10	
0.50 g	.504	.2056	.0714	1.38	.0135[f]
	±.001	±.002	±.002	±.07	±.005
2.00 g	.507	.2141	.0965	1.14	.0208[f]
	±.0003	±.004	±.01	±.03	±.002
Pellet - Mutton					
0.5 g	.502	.2172	.0968	3.82	.0145[f]
	±.001	±.004	±.007	±.07	±.006
1.0 g	.503	.2143	.0904	3.76	.0154[e]
	±.001	±.003	±.003	±.31	

[a]Values represent means and standard errors of triplicate samples of each treatment except 2.0 g *E. coprostanoligenes* which was in duplicate.
[b]Chol = cholesterol
[c]Cop = coprostanol
[d]NDA = no detectable amount
[e] = 1 sample positive
[f] = 2 samples positive

Table 3. Wet and dry weights of samples and weight of total lipid (g), cholesterol and coprostanol (μg) of fermented pork and mutton sausages after 1 month's storage after cooking - Experiment three

Treatments	Sample Weights (g)[a]		
	Wet	Dry	Lipid
Uninoculated (control)			
Pork	.503 ±.001	.2104 ±.0008	.0704 ±.001
Mutton	.504 ±.0007	.2193 ±.002	.0836 ±.003
Lactobacillus Pellet (0.05 g) Pork	.504 ±.0003	.2164 ±.005	.0663 ±.002
Lactobacillus (0.05 g) + *E. coprostanoligenes* Pellet - Pork			
0.10 g	.502 ±.001	.2134 ±.003	.0583 ±.003
0.25 g	.501 ±.001	.2416 ±.002	.0854 ±.004
0.50 g	.506 ±.0007	.2168 ±.006	.0813 ±.003
E. coprostanoligenes Pellet - Pork			
0.25 g	.504 ±.0003	.2033 ±.003	.0660 ±.0008
0.50 g	.503 ±.0007	.1924 ±.004	.0709 ±.003
2.00 g	.502 ±.001	.2081 ±.00007	.0724 ±.002
Pellet - Mutton			
0.5 g	.504 ±.0007	.2204 ±.001	.0785 ±.01
1.0 g	.504 ±.001	.2214 ±.003	.0866 ±.001

[a]Values represent means and standard errors of triplicate samples of each treatment except 2.0 g *E. coprostanoligenes* which was in duplicate.